Health Research Symposium 2017
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Health Research Symposium 2017: creating knowledge in complex system for sustainable community health

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The Health Research Symposium 2017 was held on 16 June 2017 at the Hong Kong Academy of Medicine Jockey Club Building. The Symposium was organised by the Food and Health Bureau and commemorated the 20th anniversary of the establishment of the Hong Kong Special Administrative Region. The event provided a platform to facilitate dialogue among local researchers on their latest achievements in health-related research and learn from international experiences. It aimed at setting a benchmark for excellent research in health and medicine and fostering collaboration in research to improve the health of the population. The Symposium was attended by more than 500 delegates, including 200 poster presenters. Dr Ko Wing-man, the Secretary for Food and Health, opened the Symposium by welcoming the keynote speakers, distinguished guests, and other participants. Dr Ko presented souvenirs to the keynote speakers.

The Symposium commenced with a short video 'About Health and Medical Research Fund'[1] that described the evolution and significant role played by the Health and Medical Research Fund in generating evidence-based knowledge for informing health policy and healthcare services in Hong Kong. Informative studies were highlighted and the contribution of key stakeholders was acknowledged.

Keynote Session 1 (Moderator: Prof Gabriel Leung)

Dr Douglas Bettcher

Director, Department for Prevention of Noncommunicable Diseases, World Health Organization, Geneva, Switzerland

Research on noncommunicable diseases

Dr Bettcher was unable to attend in person and provided a short video clip that was played at the Symposium.[2] Dr Bettcher noted the increasing threats posed by noncommunicable diseases, which remain the largest killer in the world. In 2015, 39 million people died needlessly from noncommunicable diseases, of which 15 million died from conditions involving modifiable risk factors such as tobacco use, unhealthy diet, lack of physical exercise, and misuse of alcohol. The World Health Organization and communities around the world can change this by focusing on prevention, best practices, and cost-effective treatment and screening. Dr Bettcher acknowledged Hong Kong's efforts to restrict tobacco use by requiring health warnings and graphic images to cover 85% of the surface area of cigarette packages, which allow consumers to make more informed health choices.

Prof Sally Wyke

Deputy Director (Social Sciences), Institute of Health and Wellbeing, The University of Glasgow, United Kingdom

Systematic development and evaluation of complex interventions to improve health: a route to success?

Prof Wyke observed that with so many interventions in healthcare it was surprising that so little attention was paid to their development, or to the feasibility of implementing them. She outlined a pragmatic guide to developing complex interventions developed by the University of Glasgow. She identified six crucial steps in designing an intervention: (1) defining and understanding the problem and its causes, (2) identifying modifiable factors, (3) deciding the mechanisms of changes, (4) clarifying how change will be delivered, (5) testing and adapting the intervention, and (6) collecting evidence for rigorous evaluation. Prof Wyke illustrated this approach with reference to the CARE Plus study in Scotland, which addressed the problem of poor health outcomes in people with multi-morbidity.

Keynote Session 2 (Moderator: Prof Francis Chan Ka-leung)

Prof Louisa Jorm

Director, Centre for Big Data Research in Health, The University of New South Wales, Australia

Big data in health and medicine: issues and challenges

Professor Jorm described how health and medical big data come from a wide variety of sources. These data are characterised as big in volume because they include large numbers of individual records and/or variables. They are also characterised by variety, as they often include structured and unstructured data such as free text or images. Additional complexity arises as data are often generated in real time. Prof

Jorm described the challenges in making use of such data, which include ensuring data quality, the increasing velocity of data flows and the rapid introduction of new medical technologies. One of the biggest challenges is the need to protect privacy and maintain confidentiality.

Dr Alain Labrique

Director, Johns Hopkins University Global mHealth Initiative, United States

What is mPossible? Leveraging mobile technology for community health and global health systems

Dr Labrique defined mHealth as a broad term to capture innovations at the intersection of mobile communications and health. As the number of mobile phone subscriptions is equal to the entire human population, it is now possible to help improve the efficiency, coverage, quality, and reach of essential maternal, newborn, and child health services, especially in hard-to-reach populations in low- and middle-income countries. Dr Labrique reported that over the past decade, hundreds of innovative projects have been tested to identify mHealth strategies that can help resolve persistent health system challenges. As the coverage of the mobile phone infrastructure increases, costs will fall and mHealth solutions will be increasingly used to strengthen public and clinical health.

Parallel Session 1: Health and Health Services (Moderator: Prof Yeoh Eng-kiong)

Dr Colman Fung Siu-cheng

In-depth cost-effectiveness study of the multidisciplinary Risk Factor and Management Programme of the Hospital Authority

The multi-disciplinary Risk Factor Assessment and Management Programme-Diabetes Mellitus (RAMP-DM) of the Hospital Authority is designed to enhance management of diabetic patients in the primary care setting. Dr Fung and colleagues evaluated the cost-effectiveness of RAMP-DM compared with usual care in the primary care setting. They found that RAMP-DM was cost-saving from both health provider and societal perspectives. This means that the cost of the RAMP-DM (including the programme cost, the subject's own costs of private healthcare utilisation as well as non-medical costs associated with the programme) were offset by the savings in public medical resources required due to reduction in complications as a result of the programme.

Dr Chang Wing-chung

Sustainability of treatment effect of a 3-year early intervention programme for first-episode psychosis in Hong Kong

Dr Chang observed that early intervention has been

shown to be better than standard care in improving clinical and functional outcomes of patients with first-episode psychosis. This study examined the sustainability of superior treatment effects of extended early intervention versus standard care on illness outcomes 2 years after the end of early intervention. They found that superior treatment effects of extended early intervention in terms of functioning, symptom severity, service utilisation, and occurrence of risk behaviours were not sustained 2 years after service withdrawal.

Dr Dorothy Chan Fung-ying

Lifestyle intervention in obese Chinese adolescents with nonalcoholic fatty liver disease: a randomised controlled trial

Dr Chan noted that the prevalence of nonalcoholic fatty liver diseases in children is increasing. This study evaluated the efficacy of a counselling-based lifestyle modification programme for such adolescents. Overall, 52 post-pubertal Chinese adolescents aged 14 to 18 years with primary obesity were randomised to either a lifestyle modification programme or usual care. The primary outcome was change of intra-hepatic triglyceride content. Ten subjects (six intervention, four control) achieved complete remission of the disease after 16 weeks. A significant reduction in body fat was seen in the intervention group compared with the control group at 16 weeks. Dr Chan concluded that a lifestyle intervention of 16-weeks was effective in reducing body fat and intra-hepatic triglyceride content.

Prof Bian Zhao-xiang

Chinese herbal medicine (Ma Zi Ren Wan) for functional constipation: a prospective, double-blinded, double-dummy, randomised, controlled study

Prof Bian noted that functional constipation was a common clinical complaint. This study evaluated the efficacy of Ma Zi Ren Wan versus first-line western therapy (Senokot) in a randomised controlled trial comprising 8-week treatment with 8-week follow-up in 843 subjects. The primary outcome was complete spontaneous bowel movement. There were no severe adverse events during treatment or follow-up. Prof Bian reported that Ma Zi Ren Wan was safe and effective for alleviating functional constipation compared with Senokot and placebo.

Parallel Session 2: Advanced Medical Research (Moderator: Prof Lau Yu-lung)

Dr Xiong Li

Autonomic dysfunction as measured by Ewing's battery test to predict poor outcome after acute ischaemic stroke

Dr Xiong observed that central autonomic dysfunction increases the risk of mortality after

stroke. This study investigated whether the severity of autonomic dysfunction as classified by Ewing's battery test could predict poor outcome after acute ischaemic stroke. Overall, 150 consecutive ischaemic stroke patients were recruited within 7 days of symptom onset. Of these, 114 (76%) were classified as having severe autonomic dysfunction by Ewing's battery test. Three months later, poor functional outcome was found in 32.5% of severe group patients compared with 13.9% of the minor group. Thus, Dr Xiong concluded that the severity of autonomic dysfunction as measured by Ewing's battery test predicted poor clinical functional outcome after acute ischaemic stroke.

Dr Maria Wong Pik
Uncovering resistant genes in epidermal growth factor receptor–mutated lung adenocarcinomas prior to targeted therapy

Lung cancer is the most lethal malignancy in the world. Lung adenocarcinomas are frequently driven by activating mutations in the epidermal growth factor receptor (EGFR). Tyrosine kinase inhibitors (TKI) are used to treat mutant cancers but drug response is impaired by the presence of resistant mutations. Dr Wong and colleagues conducted whole exome mutation profiling of 39 EGFR mutant lung adenocarcinomas and compared the TKI response pattern of 16 responders and 23 non-responders. The study found that known and candidate TKI-resistant mutations could be revealed by sequencing of pre-treatment excision specimens. The specific mutations identified in individual tumours could be useful for personalised medicine.

Dr Eddie Ma Chi-him
Persistence of ciguatera fish poisoning and its associated neurological manifestations in mice

Ciguatera fish poisoning is caused by ingesting fish contaminated with ciguatoxin and affects over 50 000 people worldwide every year. Dr Ma and colleagues investigated the neurotoxicity of pacific ciguatoxin-1 at doses relevant to human exposure on nervous system repair, functional recovery, and neurotransmitter metabolism in mice. This study provided the first evidence that persistence of pacific ciguatoxin-1 in the peripheral nervous system reduces the intrinsic growth capacity of peripheral neurons, resulting in delayed functional recovery and irreversible motor deficits after injury.

Dr Maria-Mercè Garcia-Barcelo
Uncovering genetic lesions underlying the most severe form of Hirschsprung disease by whole genome sequencing: a pilot study in eight family trios

Hirschsprung disease is a rare congenital disorder characterised by the absence of enteric neurons along a variable length of the distal intestine. Genetic variations associated with common and milder forms of the disease have been characterised, but many severely affected patients do not have mutations in known Hirschsprung disease genes. Dr Garcia-Barcelo and colleagues aimed to understand the genetic architecture underlying the disease by studying sporadic patients with severe Hirschsprung disease. Family trios (unaffected parents and affected probands) were screened by whole genome sequencing. Pathway analysis indicated that the extracellular matrix receptor pathway was significantly shared by the patients. However, the pronounced genetic heterogeneity observed indicated that genetic counselling is not advisable at this time.

Parallel Session 3: Health Promotion (Moderator: Dr Felix Chan Hon-wai)

Prof Lam Tai-hing
Promotion and brief interventions of smoking cessation at the smoking hotspots

Prof Lam and colleagues conducted a smoking cessation promotion at 14 smoking hotspots, ie outdoor areas with a large number of smokers who gather to smoke and with rubbish bins for cigarette butt disposal. These hotspots are usually located at bus stops, entrances to commercial buildings and shopping malls. Forty university student ambassadors were trained with knowledge of tobacco control, smoking cessation and techniques to approach smokers at hotspots. The ambassadors proactively delivered brief intervention, measurement of exhaled carbon monoxide level, brief advice and invitation for telephone follow-up. As a result of the promotion programme, 3096 smokers were approached. Of these, 916 received brief smoking cessation advice and 210 smokers consented to further telephone follow-up. In all 1285 smokers who received any intervention, the self-reported quit rate was 1.2%.

Ms Sania Yau Sau-wai
'We Wrap': an innovative empowerment and education programme for people with mental health challenge and young people

The Wellness Recovery Action Plan was developed in the United States as a system of physical and mental health that focuses on hope, personal responsibility, learning and maintaining your own ideas and rights. The plan focuses on training participants using specially developed materials to learn to cope with different changes in life, maintain positive thinking and mental well-being. Youth participants exposed to the programme had significantly better enhancement in hope, empowerment, mental well-being, personal confidence, willingness to ask for help, goal and success orientation, self-care and self-efficacy compared with those in the control group.

Mr Wilfred Wong Hing-sang

A geographical study of child injury in Hong Kong: spatial variation among 18 districts

This study aimed to provide a comprehensive comparison of accident and emergency department attendance rates related to child injury among 18 districts from 2001 to 2012. It also explored the relationship between child injury and socio-economic status. During the period under study, there were 742 552 child injuries leading to accident and emergency department attendance in Hong Kong resulting in direct medical costs of HK$43 million per year. There was wide variation between districts with respect to injury rate and risk of different injury type. Higher socioeconomic status was associated with lower risk of injury. The project team suggested the current injury database could be integrated with other databases that would reveal the true injury burden and allow resource planning.

Ms Sharmila Gurung

Every woman counts: cancer prevention amongst ethnic minority women

Ethnic minorities are vulnerable in Hong Kong and their health needs are often overlooked. The aim of this promotion programme was to raise breast and cervical cancer awareness amongst ethnic minority women, increase the uptake of Pap smear screening, and increase the uptake of healthy lifestyles. A group of 21 peer educators of different backgrounds, nationality, religion, and age were trained. Together these peer educators reached over 800 participants. The peer educator method was successful in increasing knowledge of cancer prevention, increasing uptake of Pap smears, exercise habit, and consumption of vegetables. In addition, 86% of participants shared the message with one to three friends.

Parallel Session 4: Infectious Diseases (Moderator: Prof Yuen Kwok-yung)

Prof Ivan Hung Fan-ngai

Efficacy of a combined influenza and 23-valent pneumococcal polysaccharide vaccines in patients with chronic illness

Pneumococcal and influenza infections can cause serious morbidity and mortality in elderly populations. Dual vaccination with 23-valent pneumococcal polysaccharide vaccine and trivalent influenza vaccine can reduce hospitalisation and death. Prof Hung and colleagues followed up two groups of subjects with chronic illness: those aged 50-64 years and those aged ≥65 years. Among those aged 50-64 years, there were fewer hospitalisations among dual vaccine recipients for respiratory, cardiovascular or cerebrovascular diseases compared with other groups. Among the elderly aged ≥65 years, there were significantly

fewer deaths, cardiovascular events, pneumonia, and intensive care unit admissions among the dual vaccine recipients compared with the other groups. Prof Hung concluded that both vaccines should be considered as part of the vaccination programme for the elderly with chronic illness.

Prof Paul Chan Kay-sheung

Human parechovirus infection in Hong Kong neonates, infants and young children

Prof Chan noted that the epidemiology of human parechovirus in Asia remains obscure. He and his colleagues determined the prevalence, seasonality, type, distribution, and clinical presentation of human parechovirus among ~3900 children aged 3 years and younger hospitalised for acute viral illness in Hong Kong. Prof Chan reported the prevalence of human parechovirus in children under 3 years as 2.3%. A clear autumn-winter seasonality was observed, with type 1 virus being most common. The clinical presentation ranged from mild gastroenteritis, upper respiratory tract infection, and febrile rash to convulsion and severe sepsis.

Dr Joseph Wu Tsz-kei

Evaluating the health economics of routine female adolescent human papillomavirus vaccination for reducing the burden of cervical cancer in Hong Kong

Infection with human papillomavirus (HPV) increases the risk for cervical and other cancers. HPV vaccines can prevent the most common types of infection. Dr Wu and colleagues evaluated the health and economic impact of routine female adolescent nonavalent HPV vaccination on reducing the burden of cervical cancer in Hong Kong. A model was developed using local epidemiological data. When the duration of vaccine protection was 20 years and vaccine uptake was 75%, it was found that for routine vaccination to be cost-beneficial the cost for fully immunising one girl would need to be lower than HK$1738 under the human capital approach and lower than HK$2499 under the quality-adjusted life-year monetisation approach.

Dr Wong Ngai-sze

Modelling the impacts of pre-exposure prophylaxis intervention on the HIV epidemic in men who have sex with men in Hong Kong

Pre-exposure prophylaxis (PrEP) is a biomedical preventive measure that could significantly reduce sexual transmission risk of HIV infection. Men who have sex with men account for a large proportion of HIV infections in Hong Kong. Dr Wong and colleagues aimed to simulate the impact of PrEP intervention through mathematical modelling. The results showed that the HIV epidemic in men who have sex with men in Hong Kong is expected to

grow. Implementation of PrEP in the community would avert new infections and control the epidemic. The degree of impact of PrEP would depend upon population coverage, adherence, affordability, public awareness and acceptance.

Award ceremony

The Symposium ended with an award ceremony to acknowledge outstanding research whose outcome has influenced health policy and practice in Hong Kong. The award recipients were as follows:

Excellent Research Awards

Principal applicant	Project title
Dr Agnes Lai Yuen-kwan The University of Hong Kong	Long-term efficacy of extended education programme on improving treatment adherence to continuous positive airway pressure in obstructive sleep apnoea
Prof Vincent Mok Chung-tong The Chinese University of Hong Kong	Amyloid burden in post-stroke dementia
Prof Cindy Lam Lo-kuen The University of Hong Kong	A study on health-related quality of life of patients with colorectal neoplasm and cost-effectiveness analysis of colorectal cancer screening in Hong Kong
Prof Anna Lee The Chinese University of Hong Kong	Anaesthesia-related complications in adult passive smokers
Prof Vincent Wong Wai-sun The Chinese University of Hong Kong	Liver fibrosis progression in patients with chronic hepatitis B: a prospective study with paired transient elastography examination
Dr Wendy Lam Wing-tak The University of Hong Kong	A longitudinal study of psychosocial needs, physical symptom distress, and psychological distress of Chinese patients with colorectal cancer

Excellent Health Promotion Project Award

Principal applicant	Project title
Prof Joseph Lau Tak-fai The Chinese University of Hong Kong	'Love others like ourselves – pass life to others' - A social marketing programme to promote organ donation among Protestant and Roman Catholic church-goers and their significant others
Dr Samuel Chu Kai-wah The University of Hong Kong	Developing an interactive social game playable on iPhones, iPads and Facebook for promoting sexuality education among youngsters

Best Poster Awards

Principal applicant	Project title
Prof Yeoh Eng-kiong The Chinese University of Hong Kong	Measuring avoidable hospital readmissions in Hong Kong using the ambulatory care sensitive conditions
Dr Cheung Siu-tim The Chinese University of Hong Kong	Drug transporter expressions associate with drug resistance and prognosis in liver cancer patients
Dr Samuel Chu The University of Hong Kong	Developing an interactive social game playable on iPhones, iPads and Facebook for promoting sexuality education among youngsters
Prof John Nicholls The University of Hong Kong	Molecular determinants of H9N2 virus haemagglutinin and neuraminidase affecting virus tropism for the human and swine respiratory tract

Prof Sophia Chan Siu-chee, Under Secretary for Food and Health, thanked the keynote speakers, moderators, judges, the speakers in the parallel sessions, and all those who had prepared posters about their work. She also thanked the delegates for attending and looked forward to meeting them again at the next Health Research Symposium.

References

1. Research Fund Secretariat. About Health and Medical Research Fund [Video file]. YouTube. Available from https://www.youtube.com/watch?v=Qe-VVJbg5Yo. Accessed 11 July 2017.
2. World Health Organization. Dr Douglas William Bettcher delivers keynote lecture at the Health Research Symposium 2017 [Video file]. YouTube. Available from https://www.youtube.com/watch?v=KlbPbW2ujGk. Accessed 11 July 2017.

Human papillomavirus infection and squamous cell carcinoma in Hong Kong: a case-control study

PKS Chan *, KF To, SH Tsang, CH Lau, WH Kwong, YHY Chan

KEY MESSAGES

1. Human papillomavirus (HPV) DNA was found in 1.5% to 7.9% of patients with oesophageal cancer in Hong Kong.

2. Viral phenotype and molecular markers did not suggest an association between HPV infection and the development of oesophageal cancer.

3. Preventative measures specific for HPV infection such as vaccination might not have an impact on controlling oesophageal cancer.

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Introduction

Globally, oesophageal cancer is the eighth most common cancer with an annual incidence of 456 000.[1] In Hong Kong, its annual incidence is 6.4 per 100 000 men and 1.3 per 100 000 women.[2] In different regions, the incidence can vary by >20-fold. The prevalence of human papillomavirus (HPV) in oesophageal cancer also varies widely and seems to be geographically linked. High-risk HPVs are associated with cervical, oropharyngeal, and anogenital cancers. Nonetheless, the role of HPV in oesophageal cancer remains in dispute. This study aimed to elucidate the role of HPV in oesophageal cancer in Hong Kong.

Methods

This multi-centre cross-sectional case-control study was conducted from April 2012 to March 2015 and was approved by the ethics committee of the participating hospitals. Patients with clinical indications who were scheduled for endoscopic examination were recruited. Those with oesophageal squamous cell carcinoma (SCC) were defined as cases, and those with non-malignant lesions or no abnormalities detected were defined as controls. Subjects with known primary cancer outside of the upper digestive tract or any known HPV-related cancers were excluded.

The quality of DNA extracted from tissue samples was assessed by amplifying a 509-bp fragment of the beta-globin gene. HPV DNA was detected by nested polymerase chain reaction (PGMY09/11 and GP5+/6+_HK52), and typed by sequencing. To analyse the oncogenic role of high-risk HPV, the most important E6/E7 mRNA species (E6*I) was measured. To assess RNA quality, extracted preparations were tested using quantitative reverse-transcription polymerase chain reaction (RT-qPCR) targeting the splicing region of mRNA encoded by the housekeeping gene RPS18.

The integration status of HPV was examined to determine the oncogenic role of HPV. Briefly, viral integration that disrupts the E2 ORF of the HPV genome results in loss of control of expression of viral oncogenes E6 and E7. By comparing the number of copies of E2 and E7 genes in a given sample, the physical status of the viral genome can be estimated. A SYBR Green-based quantitative qPCR method[3] was applied to quantify E2 and E7 gene levels. A 10-fold (≥3 threshold cycle) difference in genome copies between E2 and E7 was regarded as an indication of HPV integration.

The total viral load was measured by a SYBR Green-based quantitative qPCR targeting the E7 gene. To adjust for variation in the amount of cells collected in each sample, the normalised viral load was obtained as $(E7_{copy} / \beta\text{-actin}_{copy}) \times 2$, where $E7_{copy}$ is crude total viral load and $\beta\text{-actin}_{copy}$ is β-actin level.

The genomic DNA sequences that spanned exons 4-9 of TP53 were analysed. The CINtec p16[INK4a] monoclonal antibody Clone E6H4 (Ventana) was used for p16[INK4a] staining by immunohistochemistry.

Continuous variables were analysed using t test or Mann-Whitney U test. Categorical variables were assessed using Chi square or Fisher's exact test. A two-tailed P value of <0.05 was considered statistically significant.

Results

Of 166 patients with histologically confirmed oesophageal cancer, 143 (86.1%) were SCC, 20 (12.0%) were adenocarcinoma, one was adenosquamous cell carcinoma, one was non–small cell carcinoma, and

one was neuroendocrine carcinoma. Patients with SCC were regarded as cases. The 168 controls with no oesophageal cancer included those with gastritis (n=116, 69.0%), oesophagitis (n=22, 13.1%), polyp (n=2), candidiasis (n=1), ectopic gastric tissue (n=1), glycogenic acanthosis (n=1), herpes simplex ulcer (n=1), or intestinal metaplasia (n=1), as well as those with no abnormalities detected in the oesophagus and stomach (n=23, 13.7%). Of the 143 cases, 112 (80.1%) were male. Cancer patients were older than controls (65.0±10.0 vs 61.0±14.1 years, P=0.004).

HPV

A total of 14 samples were found to harbour HPV DNA: HPV16 (n=13) and HPV52 (n=1). The HPV DNA positive rate was comparable between cases and controls (3.5% vs 5.4%, P=0.622). Cases and controls with oesophageal samples positive for HPV were also comparable in age (61.7 vs 62.9 years, P=0.738) and male-to-female ratio (1.3:1 vs 1:1, P=0.646).

All five oesophageal SCC specimens that were positive for HPV DNA had an adequate quality of RNA, and none showed a positive signal after RT-qPCR targeting HPV16 E6*I mRNA.

All specimens showed a detectable level of E2 and were within 10-fold (ie 3 threshold cycle) of the corresponding E7 level derived from the same specimen, and none showed evidence of E2 disruption. A low-level viral load was obtained for the five HPV16 DNA-positive SCC specimens (0.06±0.04 viral copy / cell equivalent).

The five HPV-positive cases included four male smokers and one female non-smoker. TP53 mutations were found in two cases. One was a synonymous transition (CCC→CCT) at exon 5 codon 153. The other was a non-synonymous transition (TAT→TGT) at exon 6 codon 205. All these five cases were negative for p16^{INK4a} staining, HPV16 E6*I mRNA was not detected, and no evidence of E2 disruption / viral integration was observed.

TP53 and p16^{INK4a}

A total of 48 (33.6%) of the 143 patients with oesophageal SCC had TP53 mutation. All had a single point mutation, and 38 (79.2%) also had a non-synonymous mutation. The most common

TABLE 1. TP53 mutation in subjects

Frequency of detection	Exon/ codon	Base substitution	Amino acid change	Transition/ transversion	CpG island	Within DNA-binding domain	Within L2/ L3/LSH	Disruptive	Truncating
6	4/37	TCC to ACC	S37T	Transversion	Non-CpG	No	No	No	No
1	4/125	ACG to ACT	No aa change	Transversion	CpG	Yes	Yes	No	No
1	5/138	GCC to GTC	A138V	Transversion	Non-CpG	Yes	No	No	No
1	5/153	CCC to CCT	No aa change	Transition	CpG	Yes	No	No	No
1	5/156	CGC to CGT	No aa change	Transition	CpG	Yes	No	No	No
1	5/157	GTC to TTC	V157F	Transversion	CpG	Yes	No	No	No
1	5/159	GCC to CCC	A159P	Transversion	CpG	Yes	No	No	No
4	5/175	CGC to CAC	R175H	Transition	CpG	Yes	Yes	No	No
7	5/176	TGC to TTC	C176F	Transversion	Non-CpG	Yes	Yes	Yes	No
1	5/176	TGC to TAC	C176Y	Transition	Non-CpG	Yes	Yes	No	No
1	6/192	CAG to TAG	Q192 stop codon	Transition	Non-CpG	Yes	Yes	Yes	Yes
1	6/193	CAT to TAT	H193Y	Transversion	Non-CpG	Yes	Yes	Yes	No
3	6/196	CGA to TGA	R196 stop codon	Transversion	CpG	Yes	No	Yes	Yes
3	6/205	TAT to TGT	Y205C	Transition	Non-CpG	Yes	No	No	No
1	7/245	GGC to TGC	G245C	Transversion	CpG	Yes	Yes	No	No
5	7/248	CGG to TGG	R248W	Transversion	CpG	Yes	Yes	Yes	No
8	7/249	AGG to AGA	No aa change	Transition	Non-CpG	Yes	Yes	No	No
1	8/266	GGA to GTA	G266V	Transversion	Non-CpG	Yes	No	No	No
9	8/273	CGT to CCT	R273P	Transversion	CpG	Yes	Yes	No	No
1	8/281	GAC to TAC	D281Y	Transversion	Non-CpG	Yes	Yes	No	No
2	8/282	CGG to TGG	R282W	Transversion	CpG	Yes	Yes	No	No
1	8/283	CGC to CCC	R283P	Transversion	CpG	Yes	Yes	No	No

mutation was the G:C→T:A transversion type (n=19, 39.6%), which has been reported to be associated with smoking. Nonetheless, mutation was not associated with the self-reported smoking history. The prevalence of G:C→T:A transversion in smokers and non-smokers was comparable (14.4% vs 12.5%, P=0.747). The 22 TP53 mutations were scattered through exons 4-8 and were more common in exons 5, 7, and 8 (Table 1). Two cases of SCC and two cases of adenocarcinoma were positive for p16[INK4a].

Smoking, drinking, and sexual history

Smokers were more common in cases than controls (69.0% vs 28.0%, P<0.001, Table 2). Drinking ≥3 glasses of wine or beer per week regularly was more also common in cases than controls (18.2% vs 2.4%, P<0.001). HPV status was not associated with smoking or drinking habit. Furthermore, HPV infection was not associated with oesophageal SCC even after adjusting for smoking and drinking habit

(odds ratio [OR]=0.756, 95% confidence interval [CI]=0.216-2.641).

Cases and controls (9.1% vs 5.4%) as well as HPV DNA positive and negative subjects (14.3% vs 6.7%) were comparable in terms of a history of sexually transmitted disease (Table 3). Patients with oesophageal SCC were less likely than controls to report having only one or no sex partner in their lifetime (53.8% vs 72.6%, P<0.001), but were more likely to report having >10 sex partners in their lifetime (13.3% vs. 2.4%, P<0.001) [Table 3]. About 11.2% of cases reported having oral sex, and 10.5% did not respond to this question. These rates were similar to those for controls. Sexual history was not associated with HPV infection status.

HPV association with other characteristics

Respectively 80.0% and 78.3% of HPV DNA positive and negative patients with oesophageal SCC were male. Among controls, age was not associated with

TABLE 2. TP53 mutation, smoking, drinking, and sexual history according to disease and human papillomavirus (HPV) DNA status

Disease/HPV status	No. (%) of subjects		
	TP53 (non-synonymous) mutation	Ever Smokers	Regular drinkers (≥3 glasses of wine/beer per week)
Oesophageal squamous cell carcinoma (SCC) cases (n=143)	39 (27.3)	100 (69.9)	26 (18.2)
Non-cancer controls (n=168)	-	47 (28.0)	4 (2.4)
P value	-	<0.001	<0.001
HPV DNA positive (n=14)	-	5 (35.7)	1 (7.1)
HPV DNA negative (n=297)	-	142 (47.8)	29 (9.8)
P value	-	0.375	0.745
HPV DNA positive oesophageal SCC (n=5)	2 (40.0)	4 (80.0)	1 (20.0)
HPV DNA negative oesophageal SCC (n=138)	37 (26.8)	96 (69.6)	25 (18.1)
P value	0.614	1.000	1.000

TABLE 3. Sexual history according to disease and human papillomavirus (HPV) DNA status

Disease / HPV status	No. (%) of subjects					
	History of any sexually transmitted diseases	History of genital warts	Single or no sex partner in lifetime	>10 sex partner in lifetime	Ever had oral sex (yes)	Ever had oral sex (no response)
Oesophageal squamous cell carcinoma (SCC) cases (n=143)	13 (9.1)	2 (1.4)	77 (53.8)	19 (13.3)	16 (11.2)	15 (10.5)
Controls (n=168)	9 (5.4)	2 (1.2)	122 (72.6)	4 (2.4)	17 (10.1)	18 (10.7)
P value	0.201	0.871	0.001	<0.001	0.760	0.949
HPV DNA-positive (n=14)	2 (14.3)	1 (7.1)	11 (78.6)	1 (7.1)	1 (7.1)	1 (7.1)
HPV DNA-negative (n=297)	20 (6.7)	3 (1.0)	188 (63.3)	22 (7.4)	32 (10.8)	32 (10.8)
P value	0.281	0.047	0.245	0.971	0.666	0.666
HPV DNA-positive oesophageal SCC (n=5)	0 (0.0)	0 (0.0)	3 (60.0)	1 (20.0)	0 (0.0)	1 (20.0)
HPV DNA-negative oesophageal SCC (n=138)	13 (9.4)	2 (1.4)	74 (53.6)	18 (13.0)	16 (11.6)	14 (10.1)
P value	1.000	1.000	1.000	0.515	1.000	0.430

HPV status. All HPV found in cancer patients was HPV16, and also accounted for eight of nine HPV detected in controls. None of the HPV16 detected from cancer specimens showed evidence of viral integration, E6*I mRNA expression, or p16^{INK4a} expression.

Discussion

In Hong Kong, the prevalence of HPV DNA in patients with oesophageal SCC was low (1.7-7.9%), which concurs with findings reported two decades ago.[4] None of our HPV-positive cancer samples showed viral integration or E6/7 mRNA expression. A similar prevalence (2.8-9.9%) of HPV DNA in non-cancerous oesophageal tissues also suggested that HPV was a bystander rather than the culprit. Furthermore, none of the HPV DNA-positive cancers exhibited p16^{INK4a} overexpression, which is regarded as a surrogate marker for transcriptionally active E7.

Mutation status of tumour suppressor gene TP53 could be another surrogate marker of HPV involvement. E6 abrogates the function of p53, and thus bypasses the need for TP53 mutation. In this study, one of the five HPV-positive cancer samples had TP53 mutation. Although the prevalence of mutation appeared to be lower in the HPV-negative cancer group, the small number of HPV-positive cases makes comparison difficult. Of note, a study reported no association between TP53 mutation and HPV status in Hong Kong Chinese,[4] whereas another study reported a lower TP53 mutation rate in HPV-positive oesophageal cancer in Sichuan Chinese.[5]

HPV has been found in oesophageal cancer tissues for >15 years, yet its role in the aetiology remains debatable. It is plausible that certain geographically restricted environmental risk factors enhance the oncogenicity of HPV in oesophageal cells. Both higher and lower HPV positive rates are reported from areas with a high incidence of oesophageal cancer. Environmental carcinogens accumulate more preferentially in certain parts of the world.

Conclusions

In Hong Kong, HPV DNA was found in a small proportion of patients with oesophageal SCC. Virological and molecular analyses did not support an aetiological association. Preventative strategies specifically for HPV, including vaccination, might not affect the control of oesophageal cancer.

Acknowledgement

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Smoking, human papillomavirus infection, and p53 mutation as risk factors in oropharyngeal cancer: a case-control study

PKS Chan *, JSY Chor, AC Vlantis, TL Chow, SC Fung, CH Lau, FYH Ng, CS Wong

KEY MESSAGES

1. In Hong Kong, infection with high-risk human papillomavirus (HPV) over the head and neck mucosa is not uncommon.

2. The association between HPV and head and neck cancer is site-specific, and mainly confined to the oropharynx.

3. About 26% to 30% of oropharyngeal carcinoma is associated with high-risk HPV infection, mostly HPV16. Smoking that predisposes to TP53 mutation is another risk factor.

4. There is a potential to use HPV-based non-invasive methods to screen for early oropharyngeal carcinoma. Early detection of HPV-associated cancer is associated with better response to treatment and should be a public health priority.

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Introduction

Worldwide, more than 550 000 cases of head and neck squamous cell carcinoma (HNSCC) are reported annually. Infection with human papillomavirus (HPV) plays an aetiological role in a proportion of HNSCC cases, particularly those with involvement of the palatine and lingual tonsils. This study aimed to delineate the role of HPV in HNSCC in Hong Kong.

Methods

This cross-sectional case-control study was conducted from January 2012 to December 2014 and was approved by the ethics committee of the participating public hospitals. Patients with suspected benign or malignant lesions over the head and neck mucosa were recruited. Patients with nasopharyngeal carcinoma, known recurrent cancers, or metastasis from a primary site outside the head and neck region were excluded.

HPV DNA was detected by nested polymerase chain reaction and typed by sequencing. Expression of viral oncoproteins E6/E7 was examined by measuring E6*I mRNA to define the oncogenic role. Viral integration was determined by comparing the number of copies of E2 and E7 genes.[1]

Exons 4-9 of TP53 were amplified by polymerase chain reaction and sequencing. Mutations were determined based on the reference sequences available at the International Agency for Research on Cancer TP53 database (http://p53.iarc.fr).

Results

Of the 256 subjects with histologically confirmed malignant lesions, 228 (89.1%) were HNSCC, 13 (5.1%) were salivary gland malignant tumours, and 15 (5.9%) were cancers of other histological types (Table 1). In addition, 283 controls with benign or inflammatory lesions or normal tissue collected over the head and neck mucosa were divided into different groups according to the anatomic sites where tissue samples were taken (Tables 2 and 3).

Smoking

Compared with controls, subjects with malignant lesions were more likely to have a history of smoking (48.4% vs 31.4%, P<0.001, Table 1). Subjects with SCC of the oropharynx, larynx, and hypopharynx had a higher prevalence of smoking history than those with SCC of the oral cavity, lip and paranasal sinus, and those with salivary gland malignant tumour and cancer of other histological types (67.6-80.6% vs 20.0-46.2%, Table 1). Compared with controls with lesions at the same anatomic site,

TABLE 1. Prevalence of human papillomavirus (HPV) infection, TP53 mutation, and smoking status

Disease status	Male:female ratio	Mean±SD (range) age (years)	No. (%) of subjects		
			HPV DNA positive	TP53 mutation	Ever smoked
Malignant cases of the head and neck region (n=256)	2.1:1	62.7±13.5 (21-92)	27 (10.5)	52 (20.3)	124 (48.4)
Head and neck squamous cell carcinoma (SCC) [n=228]	2.1:1	62.6±13.3 (27-92)	22 (9.6)	47 (20.6)	112 (49.1)
Oral cavity SCC (n=137)	1.2:1	63.5±14.3 (27-92)	3 (2.2)	26 (19.0)	47 (34.3)
Oropharyngeal SCC (n=34)	7.5:1	56.4±13.6 (30-88)	10 (29.4)	8 (23.5)	23 (67.6)
Laryngeal SCC (n=31)	All male	64.8±9.0 (48-84)	5 (16.1)	7 (22.6)	25 (80.6)
Hypopharyngeal SCC (n=21)	4.3:1	63.0±9.4 (49-83)	3 (14.3)	4 (19.0)	16 (76.2)
Lip and paranasal sinus SCC (n=5)	0.3:1	65.2±14.5 (46-82)	1 (20.0)	2 (40.0)	1 (20.0)
Salivary gland malignant tumours (n=13)	3.3:1	61.2±13.2 (38-84)	2 (15.4)	2 (15.4)	6 (46.2)
Cancer of other histological types (n=15)	2.0:1	64.3±15.9 (21-86)	3 (20.0)	3 (20.0)	6 (40.0)
Non-malignant controls (n=283)	1.1:1	57.0±14.4 (20-91)	30 (10.6)	16 (5.7)	89 (31.4)
Oral cavity (n=139)	4.0:1	59.7±13.9 (21-91)	16 (11.5)	3 (2.2)	35 (25.2)
Oropharynx (n=42)	1.5:1	50.4±15.5 (24-81)	2 (4.8)	4 (9.5)	17 (40.5)
Larynx (n=34)	5.8:1	54.6±13.7 (28-76)	6 (17.6)	4 (11.8)	18 (52.9)
Lip and paranasal sinus (n=20)	0.7:1	62.4±10.1 (46-84)	3 (15.0)	3 (15.0)	5 (25.0)
Salivary gland (n=48)	1.2:1	54.8±14.7 (20-81)	3 (6.3)	2 (4.2)	14 (29.2)

TABLE 2. TP53 mutation status among subjects with and without malignant tumour

Disease status	No. (%) of subjects		No. (%) of subjects with any TP53 mutation								
	Any mutation	Any non-synonymous mutation	Any trans-version	Any tran-sition	Non-CpG trans-version	A:T > T:A	A:T > C:G	A:T > G:C	G:C > A:T	G:C > C:G	G:C > T:A
Head and neck cancer overall (n=256)	52 (20.3)	43 (16.8)	35 (67.3)	17 (32.7)	25 (48.1)	7 (13.5)	1 (1.9)	6 (11.5)	11 (21.2)	4 (7.7)	23 (44.2)
Control group 1 (all) [n=283]	16 (5.7)	15 (5.3)	9 (56.3)	7 (43.8)	9 (56.3)	4 (25.0)	0 (0.0)	2 (12.5)	5 (31.3)	0 (0.0)	5 (31.3)
P value	<0.001	<0.001									
Head and neck squamous cell carcinoma (n=288)	47 (20.6)	40 (17.5)	32 (68.1)	15 (31.9)	22 (46.8)	5 (10.6)	1 (2.1)	6 (12.8)	9 (19.1)	4 (8.5)	22 (46.8)
Control group 2 (all except salivary gland) [n=235]	14 (6.0)	13 (5.5)	8 (57.1)	6 (42.9)	8 (57.1)	3 (21.4)	0 (0.0)	1 (7.1)	5 (35.7)	0 (0.0)	5 (35.7)
P value	<0.001	<0.001									
Oral cavity squamous cell carcinoma (n=137)	26 (19.0)	22 (16.1)	16 (61.5)	10 (38.5)	12 (46.2)	1 (3.8)	1 (3.8)	3 (11.5)	7 (26.9)	2 (7.7)	12 (46.2)
Control group 3 (oral cavity) [n=139]	3 (2.2)	3 (2.2)	1 (33.3)	2 (66.7)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	2 (66.7)	0 (0.0)	0 (0.0)
P value	<0.001	<0.001									
Oropharyngeal squamous cell carcinoma (n=34)	8 (23.5)	7 (20.6)	4 (50.0)	4 (50.0)	3 (37.5)	1 (12.5)	0 (0.0)	3 (37.5)	1 (12.5)	0 (0.0)	3 (37.5)
Control group 4 (oropharynx) [n=42]	4 (9.5)	4 (9.5)	3 (75.0)	1 (25.0)	3 (75.0)	1 (25.0)	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	2 (50.0)
P value	0.177	0.204									
Laryngeal squamous cell carcinoma (n=31)	7 (22.6)	5 (16.1)	6 (85.7)	1 (14.3)	3 (42.9)	1 (14.3)	0 (0.0)	0 (0.0)	1 (14.3)	1 (14.3)	4 (57.1)
Control group 5 (larynx) [n=34]	4 (11.8)	3 (8.8)	3 (75.0)	1 (25.0)	3 (75.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	3 (75.0)
P value	0.406	0.463									
Head and neck cancer overall + control groups 1-5 (n=539)	**68 (12.6)**	**58 (10.8)**	**44 (64.7)**	**24 (35.3)**	**34 (50.0)**	**11 (16.2)**	**1 (1.5)**	**8 (11.8)**	**16 (23.5)**	**4 (5.9)**	**28 (41.2)**

TABLE 3. Human papillomavirus (HPV) infection status among subjects with and without malignant tumour

Disease status	No. (%) of subjects				
	HPV DNA (any type) positive	HPV DNA (high-risk types of HPV16, 31, and 52) positive	HPV16 DNA positive	HPV integration positive*	HPV E6*I mRNA positive*
Head and neck cancer overall (n=256)	27 (10.5)	22 (8.6)	20 (7.8)	14 (5.5)	11 (4.3)
Control group 1 (all) [n=283]	30 (10.6)	24 (5.8)	22 (7.8)	2 (0.7)	1 (0.4)
Head and neck squamous cell carcinoma (n=228)	22 (9.6)	17 (7.5)	16 (7.0)	12 (5.3)	10 (4.4)
Control group 2 (all except salivary gland) [n=235]	26 (11.1)	21 (8.9)	19 (8.1)	2 (0.9)	1 (0.4)
Oral cavity squamous cell carcinoma (n=137)	3 (2.2)	1 (0.7)	1 (0.7)	1 (0.7)	0 (0.0)
Control group 3 (oral cavity) [n=139]	16 (11.5)	15 (10.8)	13 (9.4)	1 (0.7)	1 (0.7)
Oropharyngeal squamous cell carcinoma (n=34)	10 (29.4)	10 (29.4)	10 (29.4)	10 (29.4)	9 (26.5)
Control group 4 (oropharynx) [n=42]	2 (4.8)	2 (4.8)	2 (4.8)	0 (0.0)	0 (0.0)
Laryngeal squamous cell carcinoma (n=31)	5 (16.1)	3 (9.7)	3 (9.7)	1 (3.2)	1 (3.2)
Control group 5 (larynx) [n=34]	5 (14.7)	1 (2.9)	1 (2.9)	0 (0.0)	0 (0.0)
Head and neck cancer overall + control groups 1-5 (n=539)	**56 (10.4)**	**46 (8.5)**	**42 (7.8)**	**16 (3.0)**	**12 (2.2)**

* HPV E6*I mRNA detection was performed for high-risk HPV types only. All samples positive for HPV E6*I mRNA were positive for integration

subjects with oropharyngeal and laryngeal SCC had a higher prevalence of self-reported history of smoking (P=0.018 for both).

TP53 mutation

A total of 40 different mutations of TP53 were found; nine located at hot-spots. Of the 40 mutations, 36 (90.0%) were non-synonymous and resulted in amino acid substitution, and five (12.5%) resulted in a stop codon. The most frequently found mutation was located at exon 4 resulting in change of codon 37 from serine to threonine (n=9), followed by mutation at codon 249 from arginine to methionine (n=8) and mutation at codon 176 from cysteine to phenylalanine (n=6). Of 68 subjects with TP53 mutations, 66 (97.1%) had mutation at one spot and two had mutations at two spots who were ex-smokers and one had laryngeal SCC and the other had oral cavity SCC.

The prevalence of TP53 mutation was higher in subjects with head and neck cancer overall (20.3% vs 5.7%, P<0.001), HNSCC (20.6% vs 6.0%, P<0.001), and oral cavity SCC (19.0% vs 2.2%, P<0.001), compared with the corresponding control groups (Table 2). Transversion mutations were about twice as common as transition mutations in terms of head and neck cancer overall (67.3% vs 32.7%), HNSCC (68.1% vs 31.9%), and oral cavity SCC (61.5% vs 38.5%). Furthermore, 77.3% of transversion mutations occurred in non-CpG sites, and G:C > T:A was the most frequent pattern of substitution. Polymorphisms (Arg/Arg, Arg/Pro, Pro/Pro) at

codon 72 were detected at similar frequencies; the cancer groups did not differ significantly to their corresponding control group.

Human papillomavirus infection

HPV DNA (all types) was found in 56 (10.4%) samples, with HPV16 the most common (n=42, 75.0%), followed by HPV6 (n=7, 12.5%), HPV11 (n=3, 5.4%), HPV31 (n=2, 3.6%), and HPV52 (n=2, 3.6%) [Table 3]. Only 16 (38.1%) of the 42 HPV16-positive samples showed integration based on the comparison between gene copies of E2 and E7. The two HPV31-positive and the two HPV52-postive samples were negative for integration. Furthermore, 12 (75.0%) of 16 samples that showed HPV16 integration were positive for HPV E6*I mRNA (Table 3).

Cases of oropharyngeal SCC showed the highest rate for high-risk HPV DNA and differed significantly to the corresponding control group (29.4% vs. 4.8%, P=0.003, Table 3). All HPV-positive oropharyngeal SCC samples were HPV16 integration positive, and in nine out of ten cases, HPV16 E*I mRNA was also detected.

One of the five high-risk HPVs identified from laryngeal SCC specimens was positive for viral integration and E6*I mRNA expression. The overall HPV DNA positive rate was 10.4% for the control groups, and was as high as 14.7% for laryngeal samples (Table 3). Of note, one specimen from the oral cavity and another specimen from the larynx in the control group were positive for HPV16 integration and E6*I mRNA.

Sexual and drinking history

A history of sexually transmitted disease was reported in 6.7% of subjects, and was significantly more common among head and neck cancer overall (9.8% vs 3.9%, P=0.006), HNSCC (10.5% vs 4.7%, P=0.017), and oropharyngeal SCC (20.6% vs 0%, P=0.002). Only 15 (2.8%) subjects reported a history of HPV disease, mainly genital warts (80%); no significant difference between subject groups was observed. Altogether, 13.0% of subjects reported no sexual partner over their lifetime; no significant difference across different groups was observed. Compared with their respective control group, patients with oropharyngeal SCC were less likely to report having just one sexual partner over their lifetime (35.3% vs 66.7%, P=0.006), whereas patients with laryngeal SCC were less likely to report having oral sex (12.9% vs 35.3%, P=0.046).

Patients with head and neck cancer overall, HNSCC, and oral cavity SCC were more likely to report a history of regular drinking than their corresponding control groups. Similar associations were obtained when the analysis was focused on heavy drinking (≥5 glasses of beer/wine or >3 glasses of cocktail per day).

Multivariate analysis

Variables with a significant (P<0.05) or close to significant (P<0.1) association with HNSCC or oropharyngeal SCC in univariate analysis were assessed using a logistic regression model. Independent risk factors for HNSCC were older age (odds ratio [OR]=1.03, 95% confidence interval [CI]=1.02-1.05 per year older), TP53 mutation (OR=3.38, 95% CI=1.71-6.66), and HPV16 infection with oncogenic phenotype (integration and E6/7 mRNA expression) [OR=9.19, 95% CI=1.13-74.68). Independent risk factors for oropharyngeal SCC were male gender (OR=4.44, 95% CI=1.45-17.28), older age (OR=1.05, 95% CI=1.01-1.09 per year older), and having ≥1 sexual partner in a lifetime (OR=4.10, 95% CI=1.35-12.42). High-risk HPV infection was an effect modifier of the association between smoking and HNSCC. Among high-risk HPV infection positive subjects, non-smokers were less likely to have HNSCC than smokers (OR=0.03, 95% CI=0.00-0.23, P<0.005). Compared with high-risk HPV infection positive ever-smokers, high-risk HPV negative ever-smokers (OR=0.29, 95% CI=0.08-1.06, P=0.06) and non-smokers (OR=0.28, 95% CI=0.07-1.06, P=0.06) were less likely to have HNSCC. An interaction between high-risk HPV infection and TP53 mutation on the association with HNSCC was suggested. Among subjects negative for high-risk HPV infection, TP53 mutation was more likely to associate with HNSCC compared with those without TP53 mutation (OR=3.35, 95% CI=1.61-7.00, P<0.005). In subjects positive for high-risk HPV, no significant difference was observed between the groups with and without TP53 mutation for the association with HNSCC.

Discussion

HPV plays an aetiological role in oropharyngeal SCC, especially those involving the palatine and lingual tonsils. The proportion of these tumours attributed to HPV varies widely across the world, and information on Southern Chinese is limited. An aetiological association with carcinoma that develops at sites other than the oropharynx is not well known.

In the current study, about 10% of samples were HPV positive of which >80% were high-risk types. Of note, less than one third of these high-risk HPV types demonstrated evidence of viral integration or oncogene E6/7 mRNA expression. Studies that only identify HPV down to the type level without verifying the viral integration or E6/7 mRNA expression status may have overestimated the role of HPV in head and neck cancer.

By taking either E6/7 mRNA expression or viral integration as an indication of oncogenic phenotype of HPV infection, we estimated that close to 30% of oropharyngeal SCC in Hong Kong may be associated with HPV. This attributed fraction is lower than that reported from Japan (50%)[2] or Beijing (40%),[3] but is close to that from Korea (32%).[4]

A meta-analysis concluded that there was a high prevalence (30%) of HPV16/18 in laryngeal cancer specimens collected from Chinese patients with an OR of 8.07.[5] In our series, 9.7% of patients with laryngeal SCC had HPV16 infection, but only one third had evidence of integration or E6/7 mRNA expression. More in-depth studies to scrutinise the role of HPV in laryngeal cancer are needed.

High-risk HPV types encode E6 protein which disrupts the normal function of p53 and thus escapes the need for TP53 mutation in cancer development. In our study, 20.3% of subjects with HNSCC were positive for TP53 mutation. The mutation rate was lower than that reported from Europe and America, but was comparable with that from Asia. The most common pattern of mutation was G:C > T:A transversion, which is known to associate with tobacco exposure. We also observed a positive association between TP53 mutation and smoking.

Conclusions

In Hong Kong, high-risk HPV infection and TP53 mutation are independent risk factors for HNSCC. The association with high-risk HPV infection was site-specific and mainly confined to the oropharynx where palatine and lingual tonsillar carcinoma develop. About 26% to 30% of oropharyngeal carcinoma may be associated with high-risk HPV

infection, mostly HPV16. Smoking that predisposes to TP53 mutation is another risk factor. High-risk HPV infection enhances the effect of smoking on HNSCC development. Subjects positive for high-risk HPV infection were less dependent on TP53 mutation as a risk factor for HNSCC.

There is a potential to use HPV-based non-invasive methods to screen for early oropharyngeal carcinoma. Early detection of HPV-associated cancer is associated with a better response to treatment and should be a public health priority.

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Epstein-Barr virus–driven promoter hypermethylated genes in gastric cancer

J Yu *, KF To, QY Liang

KEY MESSAGES

1. *Somatostatin receptor 1 (SSTR1), meiotic recombination protein 8 (REC8), and interleukin 15 receptor, alpha (IL15RA) were identified as novel Epstein-Barr virus (EBV)–driven promoter hypermethylated genes in EBV-positive AGS-EBV, compared with EBV-negative AGS.*

2. Transcriptional silence of *SSTR1* and *REC8* in EBV-associated gastric cancers is mediated by promoter methylation. This association was not observed for *IL15RA*.

3. Gain- and loss-of-function experiments revealed that *SSTR1* and *REC8* acted as tumour suppressors through modulating cell proliferation, cell cycling, apoptosis, and migration.

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Introduction

Epstein-Barr virus (EBV)–associated gastric cancer accounts for 8% to 10% of all gastric cancers. Its clinicopathological features are distinct from EBV-negative gastric cancer.[1] EBV-associated gastric cancer has been reported to involve promoter hypermethylation of tumour suppressors.[2,3] We identified *Somatostatin receptor 1 (SSTR1), meiotic recombination protein 8 (REC8), and interleukin 15 receptor, alpha (IL15RA)* to be novel EBV-driven promoter hypermethylated genes in EBV-positive AGS-EBV, compared with EBV-negative AGS.[4] *SSTR* proteins belong to the G protein-coupled receptor family and are crucial in regulating the growth inhibitory effect of somatostatin and reducing tumour cell growth. The meiotic cohesin *REC8* belongs to the cohesin protein complex, which is essential for correct chromosome disjunction and homologous recombination during mitosis and meiosis. Whether these hypermethylated genes are associated with EBV-associated gastric cancer remains elusive. In this study, we examined the epigenetic regulation, clinical significance, biological function, and molecular mechanism of these genes in gastric cancer.

Methods

Gastric cancer tissue samples were collected at the First Affiliated Hospital of Sun Yat-sen University, Guangzhou from 1999 to 2006 and Prince of Wales Hospital, The Chinese University of Hong Kong from 2005 to 2013. The presence of EBV was determined by detection of EBV-encoded RNA (EBER). This study was approved by the ethics committees of both The Chinese University of Hong Kong and the Clinical Research Ethics Committee of Sun Yat-sen University. Informed consent was obtained from each subject. The methylation status was evaluated using bisulfite genomic sequencing, combined bisulfite restriction analysis, and pyrosequencing.

Loss- and gain-of-function experiments on *SSTR1* and *REC8* were performed after knock-down by shRNA transfection and overexpression by vector transfection in gastric cancer cell lines. Cell growth was evaluated by cell viability and colony formation assay. The cell cycle distribution and apoptosis were determined by flow cytometry. Migration ability and invasiveness were assessed by wound healing and Matrigel invasion assays. Molecular mechanisms were elucidated by Human Cancer Pathway Array.

Results

SSTR1 mRNA expression was reduced or silenced in 63.4% (7/11) of gastric cancer cell lines, but readily expressed in normal gastric tissues. Promoter hypermethylation of *SSTR1* was detected in cells with *SSTR1* down-regulation (Fig 1). The promoter methylation level of *SSTR1* was higher in EBV-positive than EBV-negative gastric cancers (15.04±8.69% vs 6.93±3.01%, P=0.004). Using receiver operating characteristic (ROC) curve analysis, a cut-off value of 9.675% in *SSTR1* promoter methylation could discriminate EBV-positive from EBV-negative gastric cancers, with 75% sensitivity and 85.7% specificity (area under the ROC curve [AUC]=0.777, 95% confidence interval [CI]=0.579-0.974). *SSTR1* promoter methylation was associated with male gender (P=0.024) and EBER positive staining (P<0.005).
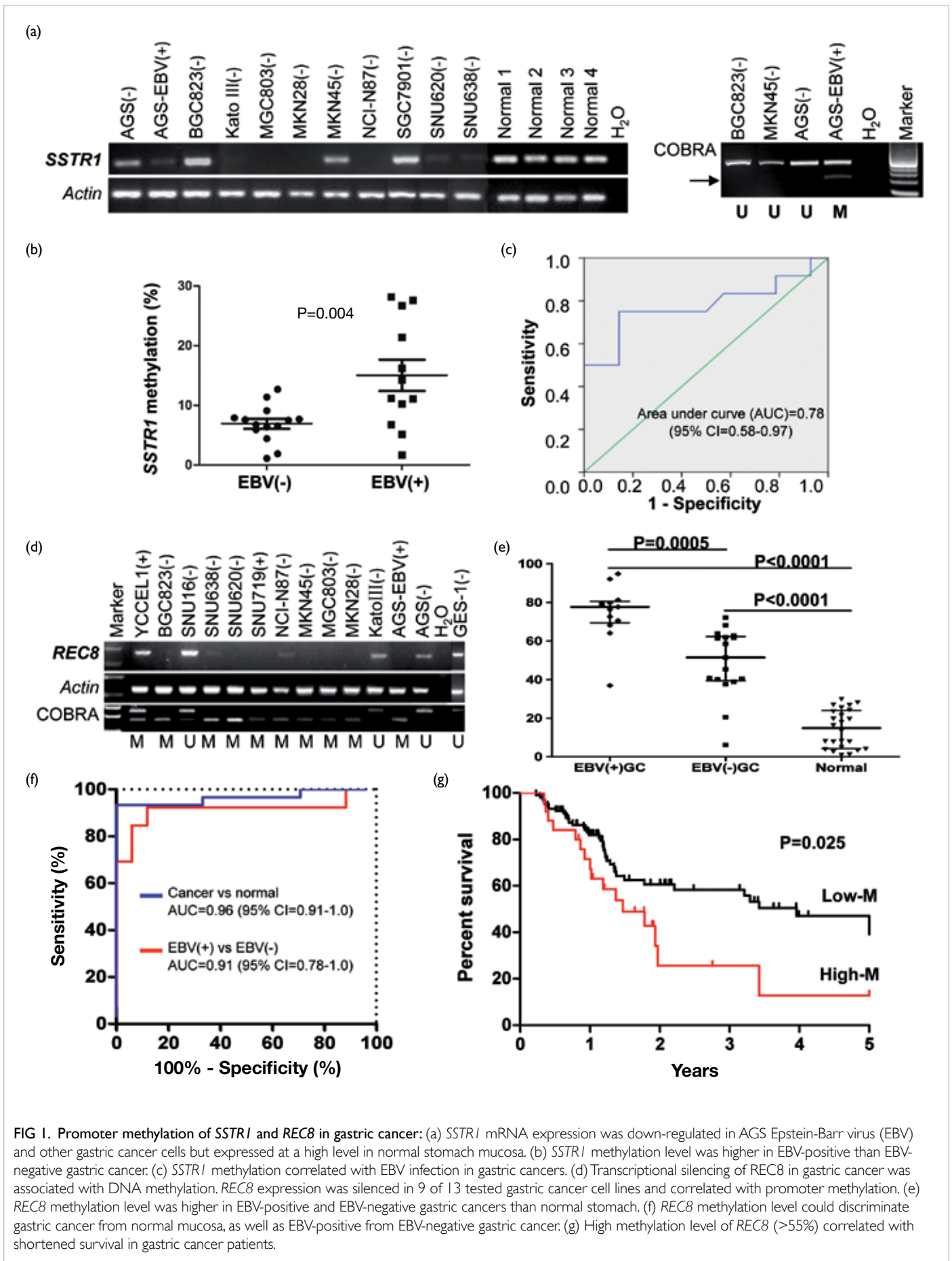
FIG 1. **Promoter methylation of *SSTR1* and *REC8* in gastric cancer:** (a) *SSTR1* mRNA expression was down-regulated in AGS Epstein-Barr virus (EBV) and other gastric cancer cells but expressed at a high level in normal stomach mucosa. (b) *SSTR1* methylation level was higher in EBV-positive than EBV-negative gastric cancer. (c) *SSTR1* methylation correlated with EBV infection in gastric cancers. (d) Transcriptional silencing of REC8 in gastric cancer was associated with DNA methylation. *REC8* expression was silenced in 9 of 13 tested gastric cancer cell lines and correlated with promoter methylation. (e) *REC8* methylation level was higher in EBV-positive and EBV-negative gastric cancers than normal stomach. (f) *REC8* methylation level could discriminate gastric cancer from normal mucosa, as well as EBV-positive from EBV-negative gastric cancer. (g) High methylation level of *REC8* (>55%) correlated with shortened survival in gastric cancer patients.

REC8 mRNA expression was silenced in 9 of 13 tested gastric cancer cell lines, and this was correlated with promoter methylation (Fig 1). Promoter methylation level of *REC8* was higher in EBV-positive than EBV-negative gastric cancers (74.8±3.9% vs 48.9±4.3%, P=0.0005), and in both gastric cancers than normal stomach mucosa (14.4±2.0%, both P<0.0001). REC8 promoter methylation was associated with EBER positive staining (r=23.73, 95% CI=10.72-36.74, P=0.001) but not with other clinicopathological features. Using ROC curve analysis, a cut-off value of 33.4% in *REC8* promoter methylation could discriminate gastric cancer from normal mucosa, with 93.3% sensitivity and 100% specificity (AUC=0.96, 95% CI=0.91-1.0). A cut-off value of 68.1% could discriminate EBV-positive from EBV-negative gastric cancer, with 84.6% sensitivity and 94.1% specificity (AUC=0.91, 95% CI=0.78-1.0). *REC8* was down-regulated by promoter methylation in gastric cancer, especially in the EBV-positive subtype. In patients with EBV-negative gastric cancer, a high level of *REC8* promoter methylation (>55%) predicted shortened survival (P=0.025).

IL15RA was down-regulated via promoter methylation specifically in AGS-EBV but this was not a common phenomenon in gastric cancer (data not shown).

To investigate the function of *SSTR1* in gastric cancer, *SSTR1* expression was knocked down by shRNA transfection in BGC823 cells as evidenced by western blot (Fig 2). *SSTR1* knock-down increased cell viability as indicated by MTS assay (P<0.05) and clonogenicity of BGC823 as indicated by colony assay (P<0.05). Cell cycle progression was promoted, with decreased cells in G1 phase (P<0.01) and increased cells in S phase (P<0.001). *SSTR1* knock-down also increased migration ability (P<0.01) and invasiveness (P<0.01) of BGC823 cells. Similar effects were also observed in AGS cells (data not shown). In gain-of-function experiments, ectopic expression of *SSTR1* in MGC803 cells was evidenced by western blot. *SSTR1* inhibited clonogenicity of MGC803 cells (P<0.01) and growth of xenograft tumours derived from MGC803 *in vivo* (n=9/group). The molecular basis of *SSTR1* was revealed by pathway cDNA array and western blot. *SSTR1* functioned as a tumour suppressor through regulating cell cycle progression, inhibiting proliferation, inducing apoptosis, and suppressing migration/invasion.

We overexpressed *REC8* in AGS-EBV cells for gain-of-function experiments. Ectopic expression of *REC8* was evidenced by western blot (Fig 3). Cell viability was decreased as indicated by MTS assay (P<0.05). *REC8* also inhibited clonogenicity of AGS-EBV cells (P<0.01) and cell cycle progression, with increased cells in G1 phase (P<0.05) and decreased cells in S phase (P<0.001). *REC8* also induced apoptosis and suppressed the migration ability of AGS-EBV cells. Similar effects were also observed in BGC823 cells (data not shown). When *REC8* expression was knocked down in GES-1 cells, cell growth was promoted as evidenced by increased cell viability and clonogenicity. *REC8* knock-down also increased cell migration ability. *REC8* also functioned as a tumour suppressor through regulating cell cycle progression, inhibiting proliferation, inducing apoptosis, and suppressing migration/invasion.

Discussion

SSTR1 promoter methylation was associated with EBV infection and may play a role in the development of EBV-associated gastric cancer. EBV infection was the only factor associated with a high *REC8* promoter methylation level. Higher *REC8* promoter methylation correlated with shortened survival in gastric cancer patients. Promoter methylation-mediated silencing of *REC8* may play a role in gastric carcinogenesis.

SSTR1 might exert its tumour suppressive function through proliferation and apoptosis regulators, inducing *MDM2, AKT, PI3KR1, BCL-XL*, and *MET*. The increased G1/S phase transition by *SSTR1* knock-down might be associated with downregulation of cyclin-dependent kinase inhibitors p15, p16, p21, and p27 and upregulation of MYC and CDC25A. The suppressive effect of *SSTR1* on migration and invasion ability might be due to the down-regulation of integrin family members (*ITGA1, ITGA2, ITGA3*, and *ITGB5* subunits), and other important migration/invasion-related genes *MMP1, PLAUR*, and *IL8*.

The anti-growth effect of *REC8* might be mediated by inhibiting the cell proliferation regulators (*G6PD* and *SLC2A1*) and apoptosis inhibitor (*NOL3*), while inducing expression of the apoptosis regulator (*GADD45G*) and tumour suppressors (*PinX1, IGFBP3*, and *ETS2*). REC8 caused cell-cycle arrest at G1/S transition through inhibiting *MCM2* and increasing *PinX1*. The anti-migration function of *REC8* may be mediated through inhibiting EMT promoters *SNAI1* and *SNAI2* (that are involved in generating de-differentiated cells) and inducing the migration inhibitor *LDHA*. Whether these cancer-associated genes modulated by altered expression of *SSTR1* or *REC8* are direct downstream targets requires further investigation.

Conclusion

Both *SSTR1* and *REC8* are novel EBV-associated promoter hypermethylated genes in gastric cancer. They play a role in suppressing gastric cancer through modulating the expression of important effectors involved in the regulation of cell proliferation,
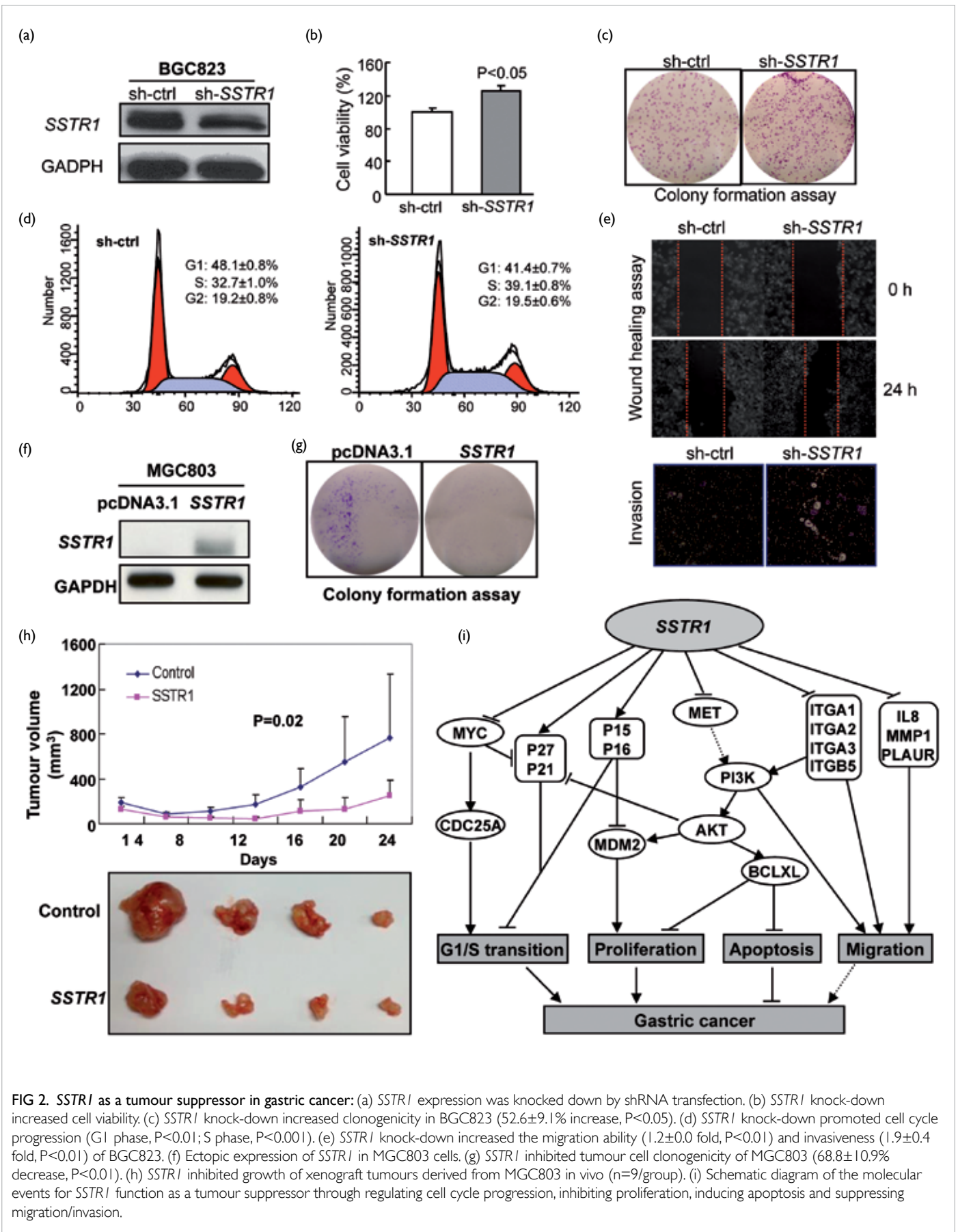
FIG 2. *SSTR1* as a tumour suppressor in gastric cancer: (a) *SSTR1* expression was knocked down by shRNA transfection. (b) *SSTR1* knock-down increased cell viability. (c) *SSTR1* knock-down increased clonogenicity in BGC823 (52.6±9.1% increase, P<0.05). (d) *SSTR1* knock-down promoted cell cycle progression (G1 phase, P<0.01; S phase, P<0.001). (e) *SSTR1* knock-down increased the migration ability (1.2±0.0 fold, P<0.01) and invasiveness (1.9±0.4 fold, P<0.01) of BGC823. (f) Ectopic expression of *SSTR1* in MGC803 cells. (g) *SSTR1* inhibited tumour cell clonogenicity of MGC803 (68.8±10.9% decrease, P<0.01). (h) *SSTR1* inhibited growth of xenograft tumours derived from MGC803 in vivo (n=9/group). (i) Schematic diagram of the molecular events for *SSTR1* function as a tumour suppressor through regulating cell cycle progression, inhibiting proliferation, inducing apoptosis and suppressing migration/invasion.
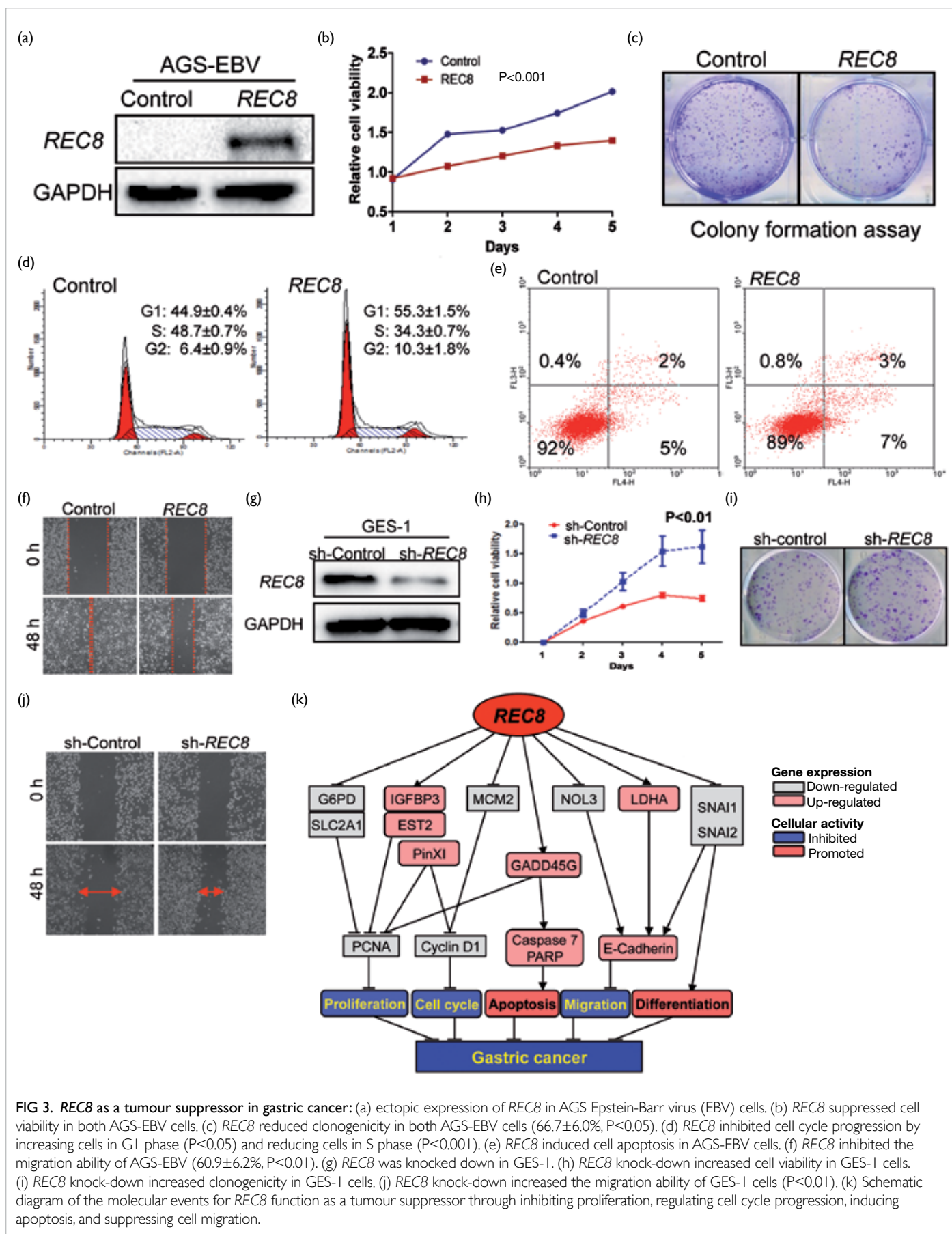
FIG 3. *REC8* as a tumour suppressor in gastric cancer: (a) ectopic expression of *REC8* in AGS Epstein-Barr virus (EBV) cells. (b) *REC8* suppressed cell viability in both AGS-EBV cells. (c) *REC8* reduced clonogenicity in both AGS-EBV cells (66.7±6.0%, P<0.05). (d) *REC8* inhibited cell cycle progression by increasing cells in G1 phase (P<0.05) and reducing cells in S phase (P<0.001). (e) *REC8* induced cell apoptosis in AGS-EBV cells. (f) *REC8* inhibited the migration ability of AGS-EBV (60.9±6.2%, P<0.01). (g) *REC8* was knocked down in GES-1. (h) *REC8* knock-down increased cell viability in GES-1 cells. (i) *REC8* knock-down increased clonogenicity in GES-1 cells. (j) *REC8* knock-down increased the migration ability of GES-1 cells (P<0.01). (k) Schematic diagram of the molecular events for *REC8* function as a tumour suppressor through inhibiting proliferation, regulating cell cycle progression, inducing apoptosis, and suppressing cell migration.

cell cycle, apoptosis, and cell migration/invasion. Epigenetic silencing of *REC8* or *SSTR1* by EBV infection may contribute to the pathogenesis of EBV-associated gastric cancer.

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Liver fibrosis progression in patients with chronic hepatitis B: a prospective study with paired transient elastography

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KEY MESSAGES

1. Liver fibrosis progression is uncommon in chronic hepatitis B patients with normal liver function tests.

2. In patients with active disease, antiviral therapy can effectively prevent fibrosis progression and even result in fibrosis regression.

3. Metabolic syndrome approximately doubles the risk of fibrosis progression.

4. Clinicians should monitor and manage metabolic disease in patients with chronic hepatitis B.

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Introduction

Chronic hepatitis B (CHB) is the leading cause of hepatocellular carcinoma (HCC) and cirrhosis in Asia. More than 400 million people worldwide are estimated to be chronically infected with hepatitis B virus (HBV). The incidence of cirrhosis and advanced liver fibrosis in patients with CHB remains unclear. Ultrasonography and liver biopsy have been used as diagnostic tools. Nevertheless, ultrasonography is operator-dependent and not sensitive to liver fibrosis or early cirrhosis. Although liver biopsy is the gold standard to assess liver fibrosis, it is invasive and may not be acceptable to many patients.

Transient elastography by Fibroscan (Echosens, Paris, France) has been developed as an accurate, reproducible, and non-invasive tool to assess liver fibrosis. By pairing transient elastography and liver biopsies in CHB patients, we developed and validated an algorithm for transient elastography to detect advanced fibrosis and cirrhosis, with area under the receiver operating characteristics curve up to 0.87 and 0.93, respectively.[1] Transient elastography can be used to perform repeated liver fibrosis assessment in a large number of patients. Patients with different disease activity, including those with inactive disease, may be studied. In the current study, we performed serial clinical assessment and transient elastography in CHB patients after an interval of ≥3 years.

Methods

From 2006 to 2008, we conducted a territory-wide screening on the prevalence of advanced liver fibrosis, in relation to the presence of metabolic syndrome, in CHB patients.[2] Over 1400 patients were recruited from primary care clinics and hospitals in Hong Kong. Patients with CHB diagnosed by positive serology tests for serum hepatitis B surface antigen (HBsAg) for at least 6 months were included. Patients with other liver diseases or liver decompensation at baseline were excluded. From 2010 to 2012, patients were invited for follow-up using transient elastography after an interval of ≥3 years. The study protocol was approved by the local ethics committee, and written informed consent was obtained from each patient before enrolment.

All patients completed comprehensive clinical and laboratory (haematological, biochemical and virologic) assessments at baseline and follow-up visits. Anthropometric parameters including body weight, body height, hip circumference, and waist circumference were measured. Serum HBV DNA levels were measured by the TaqMan real-time polymerase chain reaction assay with a range of detection of 20 to 2x10^8 IU/mL. HBsAg was quantified by Architect HBsAg QT (Abbott Diagnostic), with a final range of detection of 0.05 to 124 950 IU/mL.

According to the modified National Cholesterol Education Program criteria, diagnosis of metabolic syndrome was defined as the presence of any three of the following five factors: (1) central obesity, (2) raised concentration of triglycerides, (3) reduced concentration of high density lipoprotein-cholesterol, (4) raised blood pressure, and (5) raised fasting plasma glucose concentration ≥5.6 mmol/L or previously diagnosed type-2 diabetes mellitus.

Liver stiffness measurement (LSM) was performed using transient elastography according to the manufacturer instructions. The LSM was considered reliable only if 10 successful acquisitions were obtained with an interquartile range ≤30% of

LSM. Liver fibrosis progression was defined as a 30% increase in LSM value from baseline; regression was defined as a >30% decrease in LSM value from baseline.

The annual incidence was estimated by dividing the total number of liver fibrosis progression by the summation of person-time calculated in years. Multivariable analysis by logistic regression model adjusted for the change in serum alanine transaminase (ALT) and HBV DNA level was performed to determine the association between metabolic syndrome and its factors and liver fibrosis progression. Effect sizes were expressed in adjusted odds ratios (OR) and 95% confidence interval (CI).

Results

Of 1407 patients included, 1178 underwent follow-up assessment between 2010 and 2012. Overall, 515 patients had received antiviral therapy after the baseline visit, and 663 patients remained treatment-naïve (Fig). The mean age of the entire cohort was 46 years, and 63% were men. All patients had compensated liver disease. HBeAg was positive in 26%, and the mean HBV DNA level was 4.3 log IU/mL (Table 1).

Fibrosis progression and regression

Of 1178 patients, 972 (83%) had no or mild fibrosis at baseline. During a mean follow-up of 43±7 months, 145 (15%) had liver fibrosis progression, of whom 35 (3.6%) had progressed to advanced fibrosis. At baseline, 206 (18%) patients had advanced fibrosis

or cirrhosis. During follow-up, 139 (68%) had fibrosis regression, of whom 66 (32%) no longer had advanced fibrosis. Among patients with advanced fibrosis or cirrhosis at baseline, regression occurred in 112 of 139 (81%) patients who received antiviral therapy, compared with 27 of 57 (47%) who did not (P<0.001). Antiviral therapy was a major disease-modifier.

Metabolic syndrome

Among 663 treatment-naïve patients, 23 of 84 (22%) patients with incident metabolic syndrome developed liver fibrosis progression, compared with 84 of 663 (12%) patients in the entire cohort (P<0.001). Incident metabolic syndrome was a risk factor for liver fibrosis progression, independent of the change in serum ALT and HBV DNA level, compared with patients who did not have metabolic syndrome at both visits (adjusted OR=2.0, 95% CI=1.1-3.5, P=0.015, Table 2). Among the five metabolic factors, incident central obesity (adjusted OR=2.0, 95% CI=1.0-4.1, P=0.05) and low HDL-C level (adjusted OR=1.9, 95% CI=1.0-3.7, P=0.04) were independent risk factors for liver fibrosis progression (Table 2). Patients who had resolved metabolic syndrome or its factors, or those with persistent metabolic syndrome did not have a significantly increased risk of liver fibrosis progression. Nonetheless, there was no association between incident or resolved metabolic syndrome and liver fibrosis regression.

HBeAg-positive patients

Of 247 patients who were HBeAg positive at baseline and had reliable LSM both at baseline and follow-up, none developed hepatic decompensation during

FIG. Study flow chart
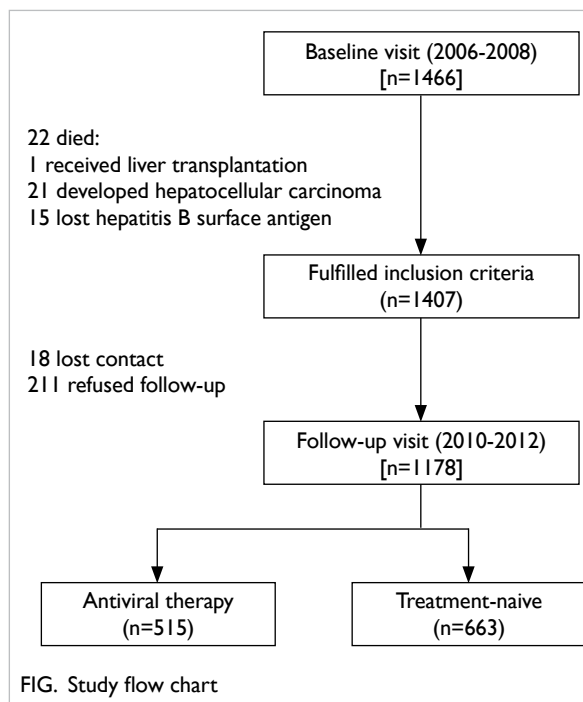
TABLE 1. Baseline characteristics of 1178 chronic hepatitis B patients

Characteristic	Value*
Age (years)	46±12
Male	743 (63)
Albumin (g/L)	44±3
Bilirubin (µmol/L)	12 (9-17)
Alkaline phosphatase (IU/L)	70 (58-89)
Alanine aminotransferase (IU/L)	45 (28-77)
Patients with positive hepatitis B e antigen	305 (26)
Hepatitis B virus DNA (log IU/mL)	4.3 (3.0-6.1)
Liver stiffness (kPa)	6.4 (4.8-8.9)
Interquartile range to median ratio of liver stiffness	0.16 (0.11-0.22)
Success rate of liver stiffness measurement (%)	91 (77-100)

* Data are presented as mean±standard deviation, median (interquartile range), or No. (%) of patients

TABLE 2. Multivariable logistic regression analysis of metabolic factors associated with liver fibrosis progression in 663 treatment-naïve patients

Status of metabolic conditions*	Incident		Resolved		Remained positive	
	Adjusted OR (95% CI)†	P value	Adjusted OR (95% CI)†	P value	Adjusted OR (95% CI)†	P value
Liver fibrosis progression						
Metabolic syndrome	2.0 (1.1-3.5)	0.015	0.9 (0.3-3.3)	0.91	0.4 (0.2-1.1)	0.09
Hypertension	0.9 (0.5-1.5)	0.67	0.8 (0.3-2.4)	0.69	1.0 (0.6-1.7)	0.99
Diabetes	1.1 (0.6-1.9)	0.82	0 (0-0)	1.00	1.1 (0.5-2.2)	0.87
Central obesity	2.0 (1.0-4.1)	0.05	1.3 (0.7-2.5)	0.42	1.4 (0.9-2.3)	0.19
Low high density lipoprotein cholesterol	1.9 (1.0-3.7)	0.04	0.8 (0.4-1.5)	0.44	0 (0-0)	1.00
High triglycerides	1.0 (0.5-1.9)	0.85	1.2 (0.5-2.9)	0.66	1.0 (0.4-2.4)	0.94
Liver fibrosis regression						
Metabolic syndrome	0.9 (0.4-1.8)	0.73	1.4 (0.5-4.1)	0.50	1.1 (0.6-2.2)	0.75
Hypertension	1.0 (0.6-1.6)	0.86	1.0 (0.4-2.8)	0.99	1.1 (0.7-1.8)	0.70
Diabetes	0.6 (0.3-1.2)	0.13	1.2 (0.3-6.3)	0.79	1.1 (0.6-2.3)	0.70
Central obesity	1.7 (0.8-3.3)	0.14	0.9 (0.5-1.8)	0.87	0.9 (0.6-1.5)	0.68
Low high density lipoprotein cholesterol	1.6 (0.7-3.1)	0.21	1.0 (0.5-1.8)	0.92	1.4 (0.4-5.3)	0.61
High triglycerides	0.7 (0.4-1.5)	0.41	1.5 (0.7-3.3)	0.33	1.4 (0.6-3.1)	0.43

* Referent to patients remained free from the metabolic conditions at both visits
† Adjusted for the change in serum alanine aminotransferase and hepatitis B virus DNA levels

follow-up and 13 (5.3%) had progressed to advanced fibrosis. The annual incidence was 1.5% (95% CI=0.8-2.6%). Among patients who remained treatment-naïve, advanced age and high HBV DNA at follow-up were associated with fibrosis progression by univariate analysis, whereas only age >40 years remained an independent factor by multivariate analysis (OR=2.1, 95% CI=1.0-5.4, P=0.05).

HBeAg-negative patients

Among 1197 HBeAg-negative patients, 361 (48±11 years of age) had reliable and normal LSM (5.4±1.5 kPa), serum ALT (28±11 IU/mL), and HBV DNA (2.7±1.0 log IU/mL) at baseline and thus were potentially inactive HBV carriers (HBsAg level, 2.5±1.4 log IU/mL). Their LSM at follow-up was 5.3±1.7 kPa. Ten patients (2.8%) had progressed to advanced fibrosis at follow-up. The annual incidence of advanced fibrosis was 0.8% (95% CI=0.4-1.4%).

Discussion

This prospective study confirmed that incident metabolic syndrome increased the risk of liver fibrosis progression in patients with CHB who remained treatment naïve after 3 to 4 years. Fibrosis progression was uncommon in untreated patients, and antiviral therapy could prevent disease progression and even result in fibrosis regression in patients with active disease.

There is an association between metabolic syndrome and cirrhosis. Insulin resistance and other metabolic disturbances can be a result and cause of cirrhosis. In our study, metabolic changes preceded fibrosis progression. This consolidated our previous observation in a cross-sectional study that metabolic syndrome increases the risk of liver cirrhosis in CHB.[2] In a Korean biopsy cohort of 850 CHB patients, metabolic syndrome was strongly associated with advanced fibrosis.[3] The increased risk of advanced liver fibrosis in patients aged >40 years who remained HBeAg-positive was probably a result of the synergistic effect of high viral load and incident metabolic syndrome, itself also increasingly prevalent with age.[4] In fact HBV infection per se may be associated with a lower prevalence of fatty liver and metabolic syndrome, and is distinct from chronic hepatitis C. The inverse association between chronic HBV infection and fatty liver was also observed in a large-scale Taiwanese population study of 33 439 subjects.[5] Nonetheless, hepatic steatosis is observed in up to 18% of CHB patients in the absence of significant alcohol consumption. Therefore concomitant non-alcoholic fatty liver disease in CHB patients remains an issue.

Advanced fibrosis is uncommon in patients in the immune-tolerant phase and inactive carrier state. Using serial transient elastography, we confirmed that fibrosis progression was also rare in these groups. We identified a subset of patients who actually had advanced fibrosis at baseline despite normal ALT and low HBV DNA level. Such patients may have been wrongly considered to be low risk cases.

Acknowledgements

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Community-based molecular epidemiology study of hepatitis C virus infection in injection drug users

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KEY MESSAGES

1. The seroprevalence of hepatitis C virus (HCV) was 76.4%. HCV-1b and HCV-6a were the two most prevalent genotypes detected in injection drug users (IDU) in Hong Kong.

2. Independent risk factors associated with HCV seropositivity were needle sharing (adjusted odds ratio [OR]=3.17), midazolam injection (adjusted OR=2.53), long duration of injection behaviour of >20 years (adjusted OR=2.45), and higher education level (adjusted OR=0.61).

3. Acute HCV infection was detected in 21 IDU, of whom 85.7% had HCV-1b infection.

4. The most recent common ancestor of the predominant HCV-6a was estimated to be around 1932, with an exponential growth of infection during 1960 to 1980.

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Introduction

Injection drug users (IDU) are at risk of hepatitis C virus (HCV) infection and account for the core of the HCV epidemic in many countries. Worldwide, >185 million individuals are estimated to be chronically infected with HCV, with Africa and Asia having the highest prevalence.[1] Persistent HCV infection is associated with cirrhosis, hepatocellular carcinoma, and HCV-related advanced liver disease. As most new cases of HCV occur among current IDU, HCV infection is a major public health problem and a growing disease burden.

The pattern of HCV epidemiology is highly variable across different countries. Egypt has the highest prevalence and incidence, with 500 000 HCV infections per year.[2] In China, approximately >40 million people are infected with HCV, and the number is increasing due to changes in the mode of HCV transmission, with a decrease in iatrogenic transmission through blood transfusion and an increase in HCV transmission through injection drug use in Southern China.[3] Globally, the distribution of HCV genotypes varies considerably between countries. This reflects differences in HCV epidemics. HCV genotypes 1, 2, and 3 exhibit broad geographical distribution and are prevalent worldwide, whereas HCV genotypes 4, 5, and 6 are generally confined to distinct geographical regions. Genotype 4 is primarily in Africa and the Middle East, genotype 5 in Southern Africa, and genotype 6 in South East Asia. HCV genotypes 1b and 2 are highly prevalent in Europe, with an increase in genotype 3a and a decrease in genotypes 1b and 2 over time. The increasing prevalence of HCV-3a is attributed to immigration and injection drug use in Eastern and Southern Europe. In China, similar changing patterns of HCV genotype distribution are also observed. The most prevalent HCV-1b has declined significantly among young individuals, whereas HCV-3b and HCV-6a have become the most common among IDU. These changing patterns of HCV infection have a significant impact on control measures and raise concern about the increasing burden of HCV infection among IDU.

The burden and transmission patterns of HCV infection in the hidden at-risk IDU population in Hong Kong remain unclear. This study aimed to assess the seroprevalence of HCV in Hong Kong IDU and to track the molecular epidemiology and transmission patterns of HCV infection over time.

Methods

This cross-sectional sero-surveillance study of HCV infection in IDU was conducted between January 2013 and June 2014. Participants were recruited through community-based outreach settings across 21 identified IDU gathering sites in Hong Kong. Participants were eligible if they were aged >18 years and reported a history of injection drug use.

A dried blood spot sample was collected

for anti-HCV and HCV RNA screening. For serological assay, anti-HCV was determined using a commercially available Murex anti-HCV version 4.0 enzyme-linked immunosorbent assay (ELISA) [DiaSorin, Italy]. Weakly reactive and positive ELISA results were confirmed by repeat screening using a Ortho HCV 3.0 Enhanced SAVe ELISA (Ortho Clinical Diagnostics, UK). For HCV RNA detection, elution from dried blood spot was performed on two 6-mm spots and was used for RNA extraction using a QIAamp viral RNA mini kit (Qiagen, USA). Reverse transcription polymerase chain reaction (PCR) with HCV primers targeting the E1 and NS5B regions was performed, followed by direct sequencing of the purified PCR amplicons. For HCV genotyping, the nucleotide sequences of the PCR products were determined directly for phylogenetic analysis using reference HCV strains retrieved from GenBank. To assess the geographic origin of HCV infection, Bayesian coalescent analysis was performed using the Bayesian Evolutionary Analysis Sampling Tree programme to reconstruct the epidemic history of HCV-6a infection in Hong Kong.

To examine the risk factors associated with HCV infection, an interviewer-administered questionnaire was completed to collect sociodemographics, duration and frequency of drug use, current injection status, injection drug use behaviours, and awareness of HCV infection. The seroprevalence of HCV infection was calculated. Categorical variables were compared using Pearson's Chi squared test or Fisher's exact test. Predictors associated with anti-HCV positivity were assessed using univariate analysis. Variables with a P value of <0.2 were included in a multivariate logistic regression model using a stepwise backward method. A P value of <0.05 was considered statistically significant.

Results

A total of 664 participants were recruited. The IDU were mostly male (87.8%), aged >30 years (96.5%), and of Chinese ethnicity (97.3%) [Table 1]. The overall HCV seropositivity among IDU in Hong Kong was 76.4% (95% confidence interval [CI]=73.1-79.6%). Acute HCV infection was observed in 21 IDU, of whom 85.7% had HCV-1b infection. Among those with acute HCV-1b infection, 11 (61.1%) were identified in a common IDU gathering site in the New Territories. HCV RNA was detected in 260 dried blood spot samples, and sequencing of the NS5B fragment was successful in 256 (98.5%) samples. Phylogenetic analysis revealed six HCV genotypes among IDU populations, with HCV-1b and HCV-6a being the most common, followed by HCV-3a, HCV-1a, HCV-2b, and HCV-3b. No co-infection was found. Based on the Bayesian coalescent analysis of E1 sequences, the epidemic

history of the divergence time of the most recent common ancestor of HCV-6a infection was estimated to be around 1932, with a transition of constant growth to exponential growth of HCV-6a during 1960 to 1980.

In univariate analysis, anti-HCV positivity was associated with age >30 years (odds ratio [OR]=2.59, 95% CI=1.03-6.44, P=0.023), male gender (OR=2.11, 95% CI=1.25-3.55, P=0.002), long duration of drug use of >20 years (OR=1.57, 95% CI=1.06-2.34, P=0.018), injection drug use of >20 years (OR=2.62, 95% CI=1.60-4.31, P<0.001), methadone treatment of at least 20 years (OR=1.55, 95% CI=1.00-2.42, P=0.040), midazolam injection (OR=2.57, 95% CI=1.72-3.83, P<0.001), needle sharing (OR=2.66, 95% CI=1.59-4.48, P<0.001), and current injection (OR=1.80, 95% CI=1.19-2.73, P=0.004). Predictor associated with decreased OR of HCV infection was observed in IDU with a higher education level (OR=0.61, 95% CI=0.42-0.89, P=0.008).

In multivariate analysis, long duration of injection behaviour of >20 years (adjusted OR=2.45, 95% CI=1.39-4.33, P=0.002), midazolam injection (adjusted OR=2.53, 95% CI=1.59-4.02, P<0.001), needle sharing (adjusted OR=3.17, 95% CI=1.73-5.81, P<0.001), and higher education level (adjusted OR=0.61, 95% CI=0.39-0.95, P=0.029) were independently associated with HCV seropositivity (Table 2).

Discussion

HCV is a major cause of chronic liver disease with most new infections attributed to injection drug use. Globally, the estimated prevalence of HCV infection among IDU is >60%. The epidemiology of HCV in Hong Kong has changed slightly over the past years. IDU remain susceptible to HCV infection as a cluster of acute HCV-1b infection has been identified in a common IDU gathering place, and possibly associated with shared social networks. In 2011, the seroprevalence of anti-HCV positivity among IDU in Hong Kong was 81.7%.[4] The decreased prevalence of HCV infection (76.4%) observed in our study could be attributed to changes in IDU behaviour, particularly a marked decline in needle sharing (6.5%) in the last 3 months.

The HCV genotype distribution is consistent with previous molecular study of HCV infection in our local IDU population.[5] In Hong Kong, genotypes 1b and 6a are more prevalent than genotype 3a. In China, there is a decreasing trend for genotype 1b and an increasing prevalence of genotype 6a. In Iran, there is a gradual decrease in the prevalence of genotype 1a and an increase in genotype 3a. The sensitivity of HCV RNA detection is markedly lower when using stored dried blood spot samples compared with plasma.[5] Due to the limitation of a relatively lower detection rate of HCV RNA,

TABLE 1. Univariate analysis of association between injection drug use behaviours and hepatitis C virus (HCV) infection

	No. (%) of subjects		OR (95% CI)	P value
	Total (n=664)	HCV seropositivity (n=507)		
Age (years)				0.023
≤30	23 (3.5)	13 (56.5)	1.00	
>30	641 (96.5)	494 (77.1)	2.59 (1.03-6.44)	
Gender				0.002
Female	81 (12.2)	51 (63.0)	1.00	
Male	583 (87.8)	456 (78.2)	2.11 (1.25-3.55)	
Ethnicity				0.778
Chinese	646 (97.3)	494 (76.5)	1.00	
Asian	18 (2.7)	13 (72.2)	0.80 (0.26-2.61)	
Education				0.008
Primary school graduate or below	382 (57.5)	306 (80.1)	1.00	
Secondary school graduate or above	281 (42.3)	200 (71.2)	0.61 (0.42-0.89)	
Duration of drug use (years)				0.018
≤20	221 (33.3)	156 (70.6)	1.00	
>20	379 (57.1)	300 (79.2)	1.57 (1.06-2.34)	
Duration of injecting (years)				<0.001
≤20	435 (65.5)	318 (73.1)	1.00	
>20	203 (30.6)	178 (87.7)	2.62 (1.60-4.31)	
Methadone treatment				0.040
≤20	433 (65.2)	322 (74.4)	1.00	
>20	198 (29.8)	162 (81.8)	1.55 (1.00-2.42)	
Midazolam injection				<0.001
No	347 (52.3)	238 (68.6)	1.00	
Yes	317 (47.7)	269 (84.9)	2.57 (1.72-3.83)	
Ever needle sharing				<0.001
No	480 (72.3)	346 (72.1)	1.00	
Yes	173 (26.1)	151 (87.3)	2.66 (1.59-4.48)	
Current injection (past 3 months)				0.004
No	161 (24.2)	109 (67.7)	1.00	
Yes	502 (75.6)	397 (79.1)	1.80 (1.19-2.73)	
Needle sharing (past 3 months)				0.143
No	597 (89.9)	447 (74.9)	1.00	
Yes	43 (6.5)	37 (86.0)	1.15 (1.01-1.31)	
Ever drug use outside Hong Kong				0.879
No	492 (74.1)	377 (76.6)	1.00	
Yes	167 (25.2)	127 (76.0)	0.97 (0.63-1.49)	
Ever injection drug use outside Hong Kong				0.333
No	525 (79.1)	397 (75.6)	1.00	
Yes	137 (20.6)	109 (79.6)	1.26 (0.77-2.04)	
Ever heard of HCV				0.936
No	117 (17.6)	89 (76.1)	1.00	
Yes	547 (82.4)	418 (76.4)	1.02 (0.62-1.67)	
Ever anti-HCV testing				0.647
No	481 (72.4)	355 (73.8)	1.00	
Yes	183 (27.6)	152 (83.1)	0.87 (0.53-1.43)	

TABLE 2. Multivariate logistic regression analysis of risk factors associated with hepatitis C virus infection

Risk factor	Adjusted OR (95% CI)*	P value
Age (reference, ≤30 years)	3.25 (0.97-10.86)	0.056
Male (reference, female)	1.23 (0.65-2.33)	0.532
Education (reference, primary school graduate or below)	0.61 (0.39-0.95)	0.029
Duration of drug use (reference, ≤20 years)	0.72 (0.43-1.20)	0.210
Duration of injecting (reference, ≤20 years)	2.45 (1.39-4.33)	0.002
Methadone treatment (reference, ≤20 years)	1.17 (0.68-2.02)	0.580
Midazolam injection (reference, never)	2.53 (1.59-4.02)	<0.001
Ever needle sharing (reference, never)	3.17 (1.73-5.81)	<0.001
Injection in past 3 months (reference, no)	1.60 (0.96-2.66)	0.071
Needle sharing in past 3 months (reference, no)	1.39 (0.35-5.50)	0.638

* Adjusted for age, gender, education level, duration of drug use, injecting behaviours, methadone treatment, midazolam injection, needle sharing, and injection and needle sharing in the past 3 months

variation in the frequency of HCV genotype over time among IDU could not be explored. Further studies of HCV RNA–based testing using freshly prepared dried blood spot are needed to track the dynamics of HCV genotype distribution and the impact on HCV transmission among IDU in Hong Kong.

In this study, phylogenetic analysis did not reveal the clustering of specific HCV genotypes, suggesting no outbreaks of HCV infection among local IDU. The association of high connectivity and risk behaviour within the HCV social networks in common IDU gathering places would play a major role in increasing HCV transmission and could impact on the rates of HCV infection and the emergence of HCV clusters.

Conclusion

Among IDU in Hong Kong, the relatively high prevalence of acute HCV-1b infection detected in a common IDU gathering site highlights the need to strengthen preventive measures and education to reduce the disease burden and improve public awareness of HCV infection.

Acknowledgements

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Epidemiology of hepatitis E infection in Hong Kong

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K E Y   M E S S A G E S

1. The overall anti–hepatitis E virus (HEV) seropositivity was 32.0%. It increased with age from 9.4% in individuals aged <30 years to 45.1% in those aged >59 years.

2. Independent risk factors associated with HEV seropositivity were age >35 years (odds ratio [OR]=3.25), no hand-washing practice after handling shellfish (OR=1.63), and higher education level (OR=0.57).

3. Anti-HEV seropositivity was more prevalent in patients with chronic liver disease (44.0%) and individuals aged >54 years (44.0%).

4. In a subgroup of frequent food handlers, the incidence of HEV infection was 0.8% during a 12-month period.

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Introduction

Hepatitis E virus (HEV) is the most common cause of enterically transmitted hepatitis worldwide and can manifest as an epidemic, particularly in Asia, Africa, and Latin America where sanitation is suboptimal. HEV infection is a major public health problem in developing countries where over 50% of acute viral hepatitis cases occur.[1] The mode of HEV transmission is mainly through the faecal-oral route, usually through contaminated food or drinking water. Although HEV infection is less common in developed countries, autochthonous HEV infection is increasingly recognised in Japan, France, the United Kingdom, and the United States, with a possible link to zoonotic transmission. Zoonotic transmission from pigs has been documented in Europe, and consumption of raw or undercooked meat from wild boar and deer has been identified as a source of zoonotic transmission of HEV in Japan.[2] Although HEV infection is often asymptomatic and self-limiting, mortality can occur particularly during pregnancy and in patients with chronic liver disease and fulminant liver failure.

The prevalence of HEV seropositivity varies between populations. In developed countries, 4% to 6% of the general population are positive for anti-HEV antibodies. Recently, higher prevalences have been reported: 16% in the United Kingdom, 21% in the United States, 29% in Germany, and 52% in southwest France. In Hong Kong, the number of HEV cases notified increased from 34 in 2006 to 150 in 2012.[3]

The burden of HEV infection in the at-risk subpopulations and the associated risk factors remain undefined. The present study aimed to assess the prevalence and risk factors of HEV infection in five subpopulations at varying risk of acquiring HEV, and to estimate the incidence of infection in the subgroup of frequent food handlers.

Methods

This cross-sectional seroepidemiological study was conducted between February 2012 and May 2014. Participants were eligible if they were aged >18 years. The study subpopulations consisted of healthy adults, pregnant women, patients with chronic liver disease, elderly people (age ≥55 years), and frequent food handlers. Pregnant women and patients with chronic liver disease were recruited from the antenatal and gastroenterology outpatient clinics, respectively. Frequent food handlers (food handling >4 days per week) included housewives, home helpers, and butchers at the wet markers.

A blood sample was collected for anti-HEV serological testing. Anti-HEV immunoglobulin (Ig) M and G were determined using commercially available enzyme-linked immunosorbent assay; positive results were confirmed by repeated serological testing. In the subpopulation of frequent food handlers, follow-up serological tests were conducted to estimate the incidence of HEV over the 12-month follow-up period. A questionnaire was used to obtain participants' sociodemographics, eating habits, food-handling practices, and knowledge and awareness of HEV infection.

Categorical variables were compared using Pearson's Chi squared test or Fisher's exact test. Association between potential risk factors and HEV

seropositivity (defined as positive for anti-HEV IgG and/or IgM) was assessed using univariate analysis. Variables with a P value of <0.2 were included in a multiple logistic regression model using the stepwise backward method. A P value of <0.05 was considered statistically significant.

Results

A total of 1539 participants, including 208 healthy adults, 215 pregnant women, 200 chronic liver disease patients, 200 elderly people, and 716 frequent food handlers were recruited. The overall HEV seropositivity was 32.0% (95% confidence interval [CI]=29.6-34.3%). The prevalence of anti-HEV increased with age (9.4% in individuals aged <30 years and 45.1% in those aged >59 years, P<0.001) and was higher in males than females (39.4% vs. 28.7%, P<0.001) [Fig 1]. In the subgroup analysis, the seroprevalence was higher among patients with chronic liver disease (44.0%, 95% CI=37.1-50.9%) and elderly people (44.0%, 95% CI=37.1-50.9%), followed by food handlers (33.5%, 95% CI=30.1-37.0%), healthy adults (20.7%, 95% CI=15.1-26.2%), and pregnant women (15.3%, 95% CI=10.5-20.2%) [Fig 2]. During the 12-month follow-up of food handlers, three cases of HEV seroconversion were identified and the incidence of HEV infection was 0.8%.

In univariate analysis, anti-HEV seropositivity was associated with older age in individuals aged >35 years (odds ratio [OR]=4.38, 95% CI=3.20-6.00, P<0.001), male gender (OR=1.61, 95% CI=1.29-2.03, P<0.001), never heard of HEV infection (OR=1.35, 95% CI=1.02-1.80, P=0.038), not knowing the animal sources of HEV infection (OR=2.23, 95% CI=1.32-3.75, P=0.003), report of no hand-washing practice after handling shellfish (OR=1.54, 95% CI=1.10-2.16, P=0.012), travel to China (OR=1.44, 95% CI=1.42-1.83, P=0.002), frequent food handling (OR=1.94, 95% CI=1.34-2.80, P<0.001), and chronic liver disease (OR=3.02, 95% CI=1.95-4.67, P<0.001) [Table]. Predictors associated with decreased OR of HEV infection were observed in individuals with a higher education level (OR=0.37, 95% CI=0.29-0.48, P<0.001) and monthly income (OR=0.67, 95% CI=0.49-0.91, P=0.011).

In multivariate analysis, age >35 years (OR=3.25, 95% CI=1.96-5.40, P<0.001), report of no hand-washing practice after handling shellfish (OR=1.63, 95% CI=1.07-2.48, P=0.023), and education level (OR=0.57, 95% CI=0.37-0.87, P=0.009) were independently associated with anti-HEV seropositivity (Table).

Discussion

In Hong Kong, the number of HEV infection cases has increased more than threefold since 2006, with 90 cases reported to the Centre for Health Protection in 2013. The epidemiology of HEV has changed over the past years, and all age groups are susceptible to HEV infection. In 2008-2009, the prevalence of anti-HEV seropositivity in Hong Kong was 28.7%.[4] The increased prevalence observed in our study suggests that exposure to HEV is common in our local populations, unlike that to hepatitis A infection.

In a community-based HEV surveillance study in a rural population in China, the risk of HEV seropositivity was higher in hepatitis B virus carriers than in the general population.[5] In our subpopulation of those with chronic liver disease, the prevalence of HEV seropositivity was 44.0%. HEV infection in the

	18-29	30-39	40-49	50-59	≥60
Overall	9.4	21.7	28.5	42.1	45.1
Male	12.3	29.0	28.4	54.1	49.0
Female	8.5	19.7	28.5	36.1	43.1
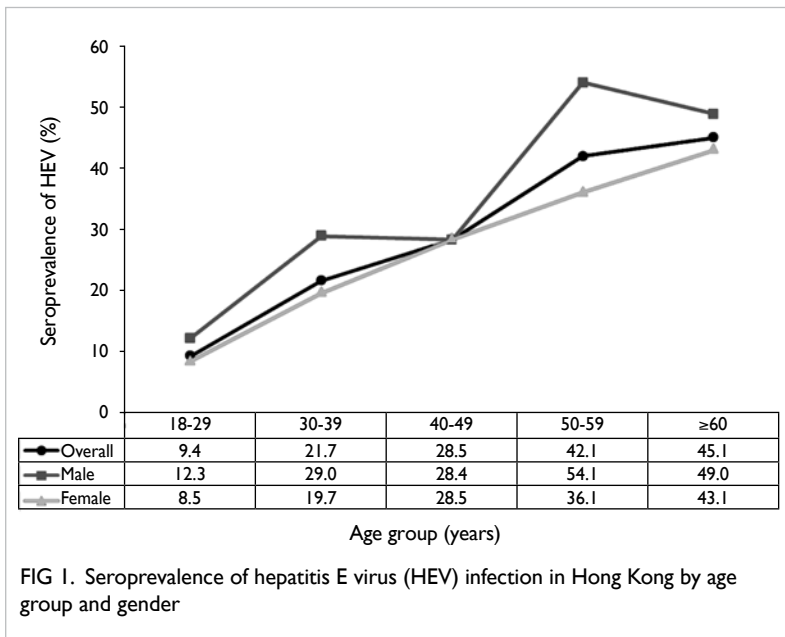
FIG 1. Seroprevalence of hepatitis E virus (HEV) infection in Hong Kong by age group and gender

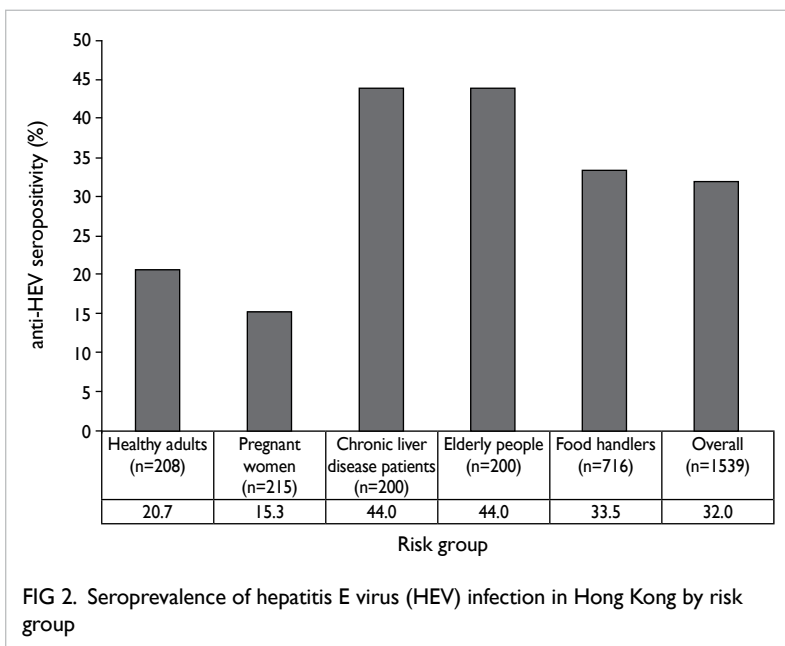| Healthy adults (n=208)	Pregnant women (n=215)	Chronic liver disease patients (n=200)	Elderly people (n=200)	Food handlers (n=716)	Overall (n=1539)
20.7	15.3	44.0	44.0	33.5	32.0

FIG 2. Seroprevalence of hepatitis E virus (HEV) infection in Hong Kong by risk group

TABLE. Predicators associated with hepatitis E virus (HEV) seropositivity in Hong Kong (n=492)

Variable	HEV seropositivity				
	No. (%) of subjects	Crude OR (95% CI)	P value	Adjusted OR (95% CI)*	P value
Demographics					
Age (years)			<0.001		<0.001
≤35	52 (10.6)	1		1	
>35	440 (89.4)	4.38 (3.20-6.00)		3.25 (1.96-5.40)	
Gender			<0.001		0.061
Female	309 (62.8)	1		1	
Male	183 (37.2)	1.61 (1.29-2.03)		1.59 (0.98-2.58)	
Level of education			<0.001		0.009
High school or less	401 (82.3)	1		1	
Above high school	86 (17.7)	0.37 (0.29-0.48)		0.57 (0.37-0.87)	
Monthly income (HK$)			0.011		0.853
≤20 000	420 (86.8)	1		1	
>20 001	64 (13.2)	0.67 (0.49-0.91)		0.947 (0.53-1.69)	
Knowledge and risk perception of HEV infection					
Ever heard of HEV infection			0.038		0.284
Yes	81 (20.6)	1		1	
No	313 (79.4)	1.35 (1.02-1.80)		1.27 (0.82-1.98)	
Vaccines are available for HEV prevention			0.172		-
Yes	41 (12.7)	1		-	
No	282 (87.3)	1.30 (0.89-1.90)			
Could identify the transmission route of HEV infection			0.696		-
Yes	3 (0.7)	1		-	
No	401 (99.3)	1.30 (0.35-4.82)			
Could identify the animal sources of HEV infection			0.003		0.909
Yes	18 (4.5)	1		1	
No	386 (95.5)	2.23 (1.32-3.75)		1.04 (0.55-1.98)	
Could identify symptoms of HEV infection			0.137		-
Yes	7 (1.7)	1		-	
No	397 (98.3)	1.88 (0.82-4.31)			
Perceived risk of HEV infection			0.795		-
High chance	276 (86.8)	1		-	
Low chance	42 (13.2)	0.95 (0.65-1.39)			
Eating habits					
Consumption of pork and pig offal			0.266		-
No	12 (2.8)	1		-	
Yes	420 (97.2)	1.45 (0.75-2.80)			
Consumption of undercooked pork and pig offal			0.710		-
No	386 (90.4)	1		-	
Yes	41 (9.6)	1.08 (0.73-1.59)			
Consumption of game meat and offal			0.766		-
No	443 (91.2)	1		-	
Yes	43 (8.8)	1.06 (0.72-1.56)			

* Adjusted for age, gender, education level, monthly income, knowledge of HEV infection, hand washing after handling shellfish, travel to China, and risk exposure groups
† Univariate logistic regression comparing with healthy adults as controls

TABLE. (cont'd)

Variable	HEV seropositivity				
	No. (%) of subjects	Crude OR (95% CI)	P value	Adjusted OR (95% CI)*	P value
Having hotpot			0.131		-
Sometimes	301 (70.3)	1		-	
Frequently	127 (29.7)	0.83 (0.65-1.06)			
Consumption of shellfish			0.085		-
No	103 (29.9)	1		-	
Yes	241 (70.1)	0.78 (0.59-1.03)			
Consumption of undercooked shellfish			0.478		-
No	264 (89.2)	1		-	
Yes	32 (10.8)	1.17 (0.76-1.82)			
Wash hands before eating			0.506		-
Yes	250 (57.9)	1		-	
No	182 (42.1)	1.08 (0.86-1.36)			
Food-handling practice					
Frequency of handling pork			0.149		-
Never	28 (7.6)	1		-	
Frequently	339 (92.4)	1.39 (0.89-2.17)			
Wash hands after handling pork			0.397		-
Yes	275 (70.3)	1		-	
No	116 (29.7)	1.12 (0.86-1.47)			
Frequency of handling shellfish			0.078		-
Never	131 (36.2)	1		-	
Frequently	231 (63.8)	0.79 (0.61-1.03)			
Wash hands after handling shellfish			0.012		0.023
Yes	155 (67.4)	1		1	
No	75 (32.6)	1.54 (1.10-2.16)		1.63 (1.07-2.48)	
Store perishable food in refrigerator and well covered			0.603		-
Yes	378 (88.5)	1		-	
No	49 (11.5)	0.911 (0.64-1.30)			
Separate raw and cooked food to prevent cross contamination			0.728		-
Frequently	332 (68.6)	1		-	
Never	152 (31.4)	0.96 (0.76-1.21)			
Risk/exposure characteristics					
Travel to HEV endemic area (China)			0.002		0.077
Never	332 (67.5)	1		1	
Frequently	160 (32.5)	1.44 (1.42-1.83)		1.49 (0.96-2.30)	
Frequent food handlers (food handling >4 days per week)†			<0.001		0.239
No	43 (15.2)	1		1	
Yes	240 (84.8)	1.94 (1.34-2.80)		0.69 (0.38-1.28)	
Pregnancy†			0.155		-
No	43 (56.6)	1		-	
Yes	33 (43.4)	0.69 (0.42-1.15)			
Chronic liver disease†			<0.001		0.240
No	43 (32.8)	1		1	
Yes	88 (67.2)	3.02 (1.95-4.67)		1.47 (0.77-2.79)	

context of liver disease carries a poor prognosis and increases the severity of the disease.

In our population, eating habits (including the consumption of pork, pig offal, and hotpot) were not associated with increased OR of HEV seropositivity. Nonetheless, multivariate analysis indicated that hand-washing practice after handling shellfish was independently associated with anti-HEV positivity, suggesting that shellfish is a potential source of foodborne HEV transmission. Further studies are needed to determine the prevalence of HEV in the food chain and to provide advice about processing of shellfish to reduce the risk of HEV infection.

Conclusion

The rising prevalence of HEV infection highlights the need to strengthen preventive measures and education to reduce the disease burden and improve public awareness of HEV infection.

Acknowledgement

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Sonodynamic bactericidal efficacy of hypocrellin A and B against methicillin-resistant *Staphylococcus aureus*

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KEY MESSAGES

1. Hypocrellin A and B have significant bactericidal activity against methicillin-resistant *Staphylococcus aureus* (MRSA).

2. Sonodynamic treatment of hypocrellin A/B inhibits protein synthesis of MRSA.

3. Hypocrellin A/B–mediated sonodynamic action causes notable damage to membrane integrity, membrane potential, and ultrastructure of MRSA.

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a public health problem. Current antibiotics have limited efficacy against MRSA and may cause multi-drug resistance.[1] There is a need to develop novel strategies to combat MRSA. This study aimed to evaluate the sonodynamic bactericidal efficacy of hypocrellin A and hypocrellin B against MRSA.

Methods

This study was conducted from April 2012 to September 2014. The MRSA (ATCC BAA-43) strains were provided by the Department of Microbiology, The Chinese University of Hong Kong.

Bacterial suspensions were incubated with hypocrellin A/B at a series of concentrations in the dark at room temperature for different lengths of time. Cell suspensions were spread on Mueller-Hinton agar and cultured for further 24 hours at 37°C. Growth of MRSA was evaluated using colony forming unit (CFU) assay and expressed as CFU/mL ($\log_{10}$).

To measure the uptake of hypocrellin A/B in MRSA, bacterial suspensions were incubated with hypocrellin A/B (50 µM). Fluorescence of samples was measured every 10-minutes after addition of sensitisers. The fluorescence of hypocrellin A/B in MRSA was measured using fluorescent analysis on a microplate reader.

To evaluate the sonodynamic bactericidal efficacy of hypocrellin A/B against MRSA, the treated bacteria were spread on Mueller-Hinton agar and incubated for 24 hours. The growth of bacteria was evaluated using the CFU assay and described as dark toxicity. The ultrasound exposure system has been described in our previous study.[2]

To investigate mechanisms of sonodynamic bactericidal activity of hypocrellin A/B against MRSA, the ultrastructural morphology of the treated bacteria was observed using a transmission electron microscope. Bacterial DNA fragmentation was measured using pulsed-field gel electrophoresis with CHEF DR-III apparatus; bacterial DNA and RNA synthesis was measured using a microplate reader in combination with chemiluminescent bromodeoxyuridine enzyme–linked immunosorbent assay and flow cytometry with Click-iT RNA Alexa Fluor 488 HCS Assay, respectively; bacterial protein synthesis was measured using a flow cytometer with Click-iT Plus OPP Protein Synthesis Assay Kit; and bacterial membrane integrity and membrane potential were measured using a flow cytometer with propidium iodide and carbocyanine dye 3,3'-diethyloxacarbocyanine iodide, respectively.

Results

Hypocrellin A/B treatment alone had no significant effect on the growth of MRSA in terms of dark toxicity. The uptake of hypocrellin A/B increased with prolonged incubation time and reached a peak after 50 minutes. The growth of MRSA was inhibited after ultrasound sonication in the presence of 80 µM hypocrellin A (P<0.0001) or 20 µM hypocrellin B (P<0.0001). There was a 5-log reduction in CFUs following ultrasound treatment in the presence of 40 µM hypocrellin B. Sonodynamic treatment of hypocrellin A/B caused notable damage to the ultrastructure of MRSA, including septal deformations, loss of the septal midline, and cell lysis.

An incomplete cell envelope was found in bacteria treated by hypocrellin B and ultrasound together. There was no significant change to chromosomal DNA in any group. There was no significant change to DNA synthesis in the combined treatment groups versus control group. DNA and RNA synthesis was comparable among the ultrasound treatment alone group, hypocrellin A treatment alone group, hypocrellin B treatment alone group, and control group. For protein synthesis, combined treatment with ultrasound and hypocrellin had more effect than ultrasound treatment alone or hypocrellin treatment alone (P<0.05). Sonodynamic treatment of hypocrellin A/B had more effect on membrane integrity than ultrasound treatment alone (P<0.05). Sonodynamic treatment of hypocrellin A/B had more effect on bacterial membrane potential than ultrasound treatment alone and hypocrellin treatment alone (P<0.05).

Conclusion

Sonodynamic treatment of hypocrellin A/B had significant bactericidal activity against MRSA. There was notable damage to the bacterial membrane caused by the sonodynamic antibacterial action of hypocrellin A/B.

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Synergists from *Portulaca oleracea* with macrolides against methicillin-resistant *Staphylococcus aureus* and related mechanism

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KEY MESSAGES

1. Two unsaturated fatty acids—linoleic acid and oleic acid—were identified from *Portulaca oleracea* that acted synergistically with erythromycin in vitro against methicillin-resistant *Staphylococcus aureus* RN4220-pUL5054, possibly by inhibiting the methionine sulfoxide reductase A multidrug efflux pump of the bacteria.

2. By comparing a panel of linoleic acid and oleic acid analogues, unsaturated fatty acids in salt form with cis configuration and an increase in number of double bonds were found to increase the antibacterial activity against RN4220-pUL5054 when used alone or in combination with erythromycin.

3. The salt forms of linoleic acid and oleic acid with erythromycin could significantly reduce the bacterial count in the lungs of mice infected with RN4220-pUL5054.

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Introduction

Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) remain a major problem worldwide. The pharmaceutical arsenal available to control MRSA is limited. Vancomycin is the mainstay treatment for MRSA, but its overuse has generated fully resistant MRSA strains. Screening of natural products for antibacterial effects, especially efflux pump inhibitors, is attracting increasing attention. Using high-speed counter-current chromatography, 18 fractions were obtained by fractionation of *Portulaca oleracea* (PO). The non-polar fraction no.18 (F18) showed synergistic activity with four antibiotics (ampicillin, ciprofloxacin, erythromycin, and gentamicin) against intermediate level vancomycin-resistant MRSA strain ST239. F18 only exhibited synergism with erythromycin against MRSA RN4220/pUL5054 that the methionine sulfoxide reductase A (MsrA) is resistant to 14-membered macrolides via an adenosine triphosphate (ATP)–binding cassette pump, and could lower the minimal inhibitory concentrations (MIC) of erythromycin by three-fold at 32 µg/mL.[1] No synergism of F18 was found against *S aureus* NorA 1199B (resistant to quinolones and harbouring a multidrug resistance pump) or aminoglycoside-resistant MRSA strains APH2"–AAC6', APH3', and ANT4'. These results suggest that F18 may contain putative efflux pump inhibitors and restore the antibacterial activity of erythromycin by inhibiting the ATP-binding cassette efflux pump but not NorA from MRSA. This study aimed to identify the active ingredients from F18 of PO that synergise with macrolides against MRSA and to determine the mechanisms of resistance and to confirm their synergistic effects with antibiotics in a murine pneumonia model.

Methods

The study was conducted from April 2012 to March 2014. The ethanol extract of PO was fractionated using a high-speed counter-current chromatography TBE-1000. MRSA strains of ST239, 1199B, RN4220-pUL5054, APH2"-AAC6', APH3', and ANT4' were used. ATCC25923 served as a control. To identify the synergistic interaction of PO active ingredients with erythromycin against SA-RN4220/pUL5054, checkerboard arrays with multiple dilution combinations of two different antimicrobial agents in a concentration range from below to above the MIC were performed in a 96-well microtitre for 24 hours at 37°C. An efflux inhibitory assay was performed to confirm whether the synergistic mechanisms of the PO-isolated compounds were mediated through inhibition of *S aureus* efflux pumps. A murine lung infection model was used to validate the *in vivo* efficacy of the isolated compounds of PO that showed promising activity against MRSA. The animal study protocols were approved by the Animal Experimentation Ethics Committee of

The Chinese University of Hong Kong (reference: 11/055/MIS).

Results

Synergism of linoleic acid and oleic acid with erythromycin against RN4220-pUL5054

Eighteen fractions were obtained by fractionation of PO. A synergistic effect was observed when F18 (32 µg/mL) was combined with erythromycin (32 µg/mL). Growth of the ATP-binding cassette efflux pump of overexpressed strain of RN4220-pUL5054 was inhibited with a fractional inhibitory concentration index (FICI) of 0.38. Using gas chromatography mass spectrometry, 13 compounds were identified. When combined with erythromycin, both linoleic acid and oleic acid inhibited the growth of the bacteria synergistically. Linoleic acid at 16 µg/mL and oleic acid at 32 µg/mL could reduce the MIC of erythromycin from 256 to 16 µg/mL (8-fold reduction) and from 256 to 32 µg/mL (4-fold reduction), respectively. The respective FICI were 0.12 and 0.18, and the respective estimated percentage yields in PO extracts were 0.093 and 0.043%.

Structural activity of linoleic acid and oleic acid analogues against RN4220-pUL5054

To explore the structural activity of the synergistic antibacterial activity of fatty acids, a panel of fatty acid analogues related to oleic acid and linoleic acid was selected for checkerboard assay using RN4220-pUL5054 (Table).[2] Sodium linoleate and sodium oleate were more potent than linoleic acid and oleic acid in inhibiting the growth of RN4220/pUL5054, and acted synergistically with erythromycin, probably due to better water solubility of these salt forms. These results suggest that improved solubility of linoleic acid and oleic acid in the form of ionic salt might enhance their antibacterial activity. When oleic acid was in an ester form, ie methyl or ethyl oleate, antibacterial activity was abolished (Table).[2] The influence of the stereochemistry was investigated. Elaidic acid, the stereoisomer of oleic acid in *trans* configuration, was not active against RN4220-pUL5054 (FICI>0.5). Similar results were also observed for vaccenic acid in *cis* and *trans* configurations, a fatty acid with 18 carbons, as in

TABLE. Minimum inhibitory concentration (MIC) and fractional inhibitory concentration index (FICI) of oleic acid analogues and related compounds with erythromycin against methicillin-resistant *Staphylococcus aureus* RN4220/pUL5054 (n=2). FICI ≤0.5 is defined as synergistic, >0.5 to ≤1.0 as additive, >1.0 to ≤2.0 as indifferent, and >2.0 as antagonistic.

Chemical structure of the fatty acid	Agent	MIC		FIC	FICI/ outcome
		Alone	Combined		
	1. Linoleic acid	256/256	16/16	0.06/0.06	0.12/0.12
	Erythromycin	256/256	16/16	0.06/0.06	Synergy
	2. Sodium linoleate	128/128	8/8	0.06/0.06	0.07/0.07
	Erythromycin	256/256	1/1	0.01/0.01	Synergy
	3. Sodium oleate	128/128	4/4	0.03/0.03	0.06/0.06
	Erythromycin	256/256	8/8	0.03/0.03	Synergy
	4. Methyl oleate	>256/>256	256/256	0.5/0.5	1.5/1.5
	Erythromycin	256/256	256/256	1.0/1.0	Indifferent
	5. Ethyl oleate	>256/>256	256/256	0.5/0.5	1.5/1.5
	Erythromycin	256/256	256/256	1.0/1.0	Indifferent
	6. Oleic acid,	>256/>256	32/32	0.06/0.06	0.12/0.18
	Erythromycin	256/256	16/32	0.06/0.13	Synergy
	7. Elaidic acid,	>256/>256	256/256	0.5/0.5	0.75/0.75
	Erythromycin	256/256	64/64	0.25/0.25	Additive
	8. cis-Vaccenic acid,	>256/>256	32/64	0.06/0.12	0.09/0.15
	Erythromycin	256/256	8/8	0.03/0.03	Synergy
	9. trans-Vaccenic acid	>256/>256	128/128	0.25/0.25	0.5/0.5
	Erythromycin	256/256	64/64	0.25/0.25	Synergy
	10. Palmitoleic acid,	256/256	16/16	0.06/0.06	0.09/0.09
	Erythromycin	256/256	8/8	0.03/0.03	Synergy
	11. Arachidonic acid	32/32	8/8	0.25/0.25	0.28/0.28
	Erythromycin	256/256	1/1	0.03/0.03	Synergy

oleic acid, but a different unsaturation position (6 and 11, respectively). The MIC of the *cis* form of vaccenic acid was two to three fold lower than that of the *trans* isomer. The MIC and FICI values of oleic acid and vaccenic acid against RN4220/pUL5054 were similar, suggesting that the position of a double bond was not a critical factor that would affect the antibacterial activity of unsaturated fatty acids. Nonetheless, the presence of a double bond appeared to be essential for antibacterial activity. Arachidonic acid, with four unsaturations, was the most potent fatty acid among all tested compounds against RN4220/pUL5054 (MIC alone, 32 µg/mL; FICI=0.25, Table).[2]

Resistance inhibition and cytotoxicity of linoleic acid and oleic acid

In the ethidium bromide efflux inhibitory assay, both sodium linoleate and sodium oleate (32 and 64 µg/mL, respectively) inhibited the fluorescence loss after 30 minutes of incubation and washing, compared with the drug-free control (Fig 1).[2] The inhibitory effects of sodium linoleate (64 µg/mL) were comparable to the positive control reserpine (64 µg/mL). When the fluorescence signals of the compounds over time were quantitatively expressed as the area under the curve (Fig 1), the values of both sodium linoleate and sodium oleate were significantly larger than the drug-free control. In contrast, palmitic acid (PA) had no significant modulating effect on the fluorescence signal of ethidium bromide–loaded cells. Neither linoleic acid nor oleic acid was toxic to human peripheral mononuclear cells, human Caco2, or human skin fibroblasts at concentrations of 32 to 128 µg/mL (data not shown).

Animal studies

In a murine pneumonia model, the amount of inoculated RN4220-pUL5054 for infection was 1×10^8 colony-forming units (CFU) per mouse. Symptoms of severe illness such as lethargy, hunched posture, ruffled fur, and weight loss were observed after infection. The mean log CFU value for all treatment groups was reduced when compared with no treatment group (8.03±0.21, Fig 2). Vancomycin was the most efficacious among all tested agents (4.73±0.08), with a >3 log reduction in bacterial counts. Since suboptimal dosages of sodium linoleate, sodium oleate and erythromycin were used due to toxicity and solubility issues, their antibacterial activity was mild. When combined erythromycin with sodium linoleate or sodium oleate, the *in vivo* antibacterial activity against RN4220-pUL5054 was enhanced, and their mean log CFU values were 6.51±0.15 and 5.80±0.14, respectively, with an almost 2-log reduction in bacterial count when compared with the no treatment group. Tissue sections from lungs infected with RN4220-pUL5054 revealed recruitment of leukocytes, inflammation of the lung parenchyma, bronchial epithelial damage, and tissue necrosis, compared with a phosphate-buffered saline control. Figure 2 shows the histological changes in the lungs of mice treated with sodium linoleate and erythromycin, sodium oleate and erythromycin, and vancomycin alone. The tissue profile of these treatment groups revealed fewer lesions, infiltration of leukocytes and erythrocytes in the airspace, and preserved alveolar structure. For mice treated with sodium linoleate, sodium oleate and erythromycin alone, the improvement in tissue profile was less significant.
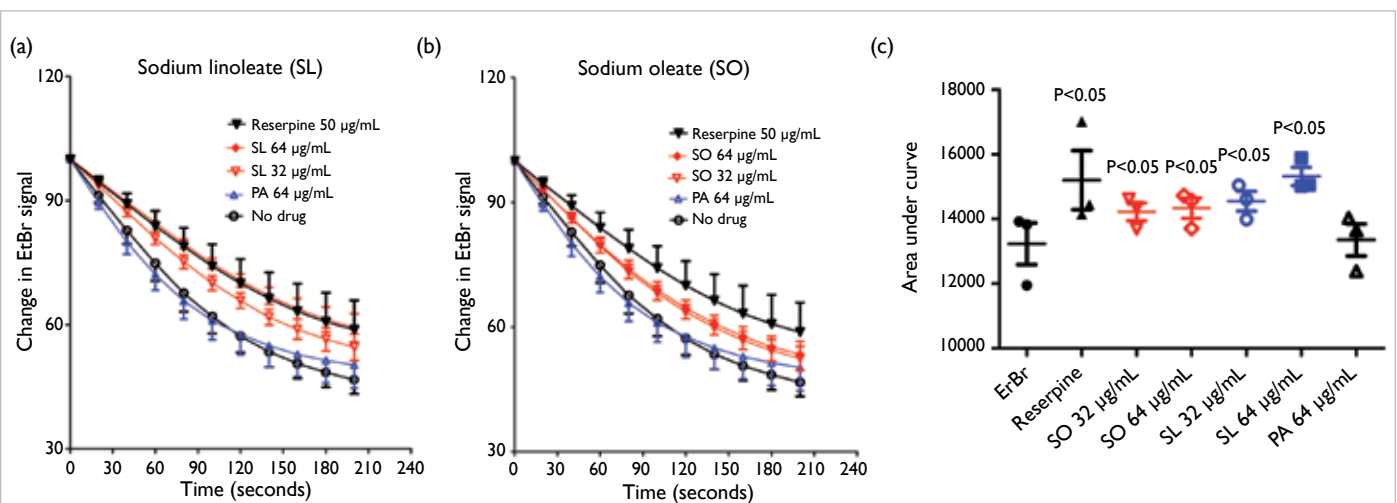

FIG 1. Effects of (a) linoleic acid and (b) oleic acid on ethidium bromide efflux (EtBr) from methicillin-resistant *Staphylococcus aureus* RN4220/pUL5054. Reserpine at 50 µg/mL was used as a positive control, and palmitic acid (PA) was used as negative a control. (c) Area under the curve of the fluorescence against time of sodium linoleate (SL), sodium oleate (SO), reserpine, and PA was compared with the drug-free control. Data are expressed as mean±standard error of mean (n=3).
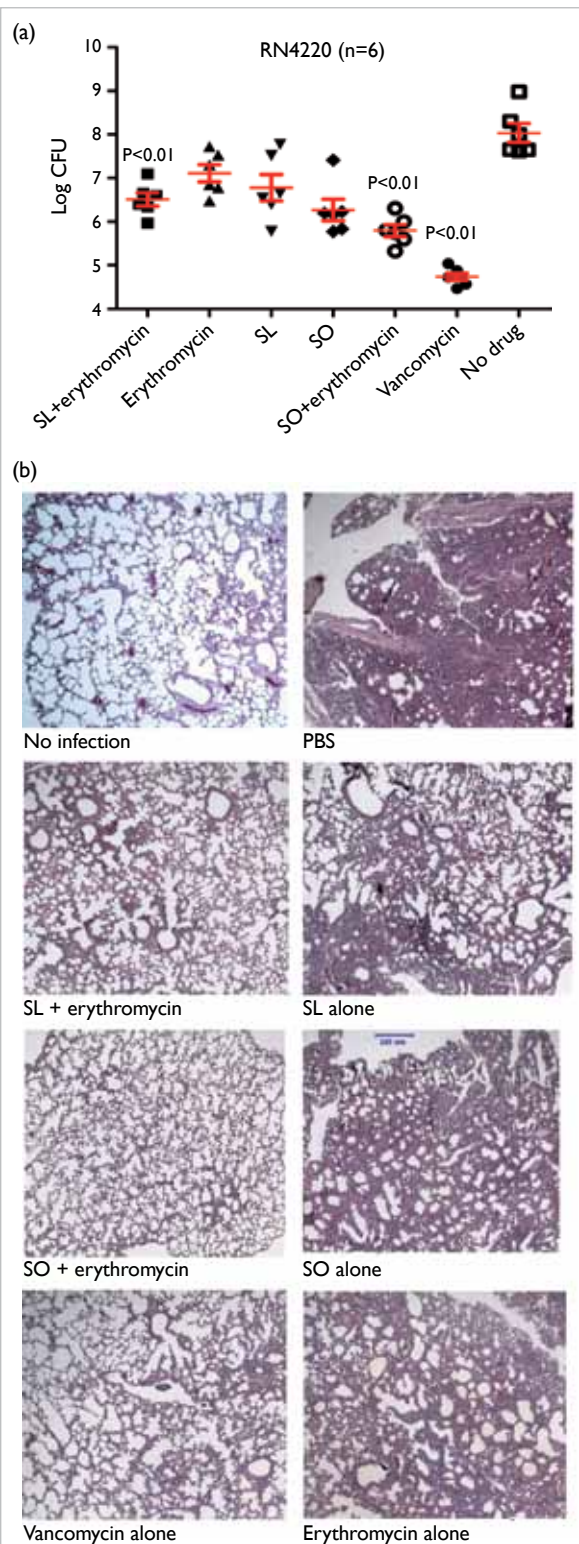
FIG 2. (a) Bacteria counts in log colony forming units (CFU) recovered from the left lung of mice infected with RN4220-pUL5054 ($1×10^8$ CFU) and treated with sodium linoleate (SL) and erythromycin, erythromycin alone, sodium linoleate (SL) alone, sodium oleate (SO) alone, sodium oleate (SO) and erythromycin, vancomycin alone, or phosphate-buffered saline (no drug) for 48 hours. (b) Histology of lung tissue in normal mice without infection, and mice infected with RN4220-pUL5054 and treated with phosphate-buffered saline (PBS), SL + erythromycin, SL alone, SO + erythromycin, SO alone, vancomycin alone, or erythromycin alone.

Discussion

We identified two unsaturated fatty acids, namely linoleic acid and oleic acid, from PO F18 with mild antibacterial activity against msrA overexpressed RN4220-pUL5054. The synergistic antibacterial activity was observed when combined with erythromycin. Linoleic acid, with two carbon double bonds, is more active than oleic acid that has one carbon double bond in its fatty acid chain.

With regard to the mechanism of resistance, both fatty acids may interfere with the msrA pump and restore the antibacterial effects of erythromycin against RN4220-pUL5054. MsrA is a 488-amino-acid protein with two ATP-binding motifs and functions independently when cloned in SA-RN4220. Linoleic acid and oleic acid may interfere with the activity of the MsrA pump and restore the activity of erythromycin. In a systematic study of the antibacterial effect of fatty acids against *S aureus* RN4220,[3] oleic acid was not active against the growth of RN4220 when used alone, whereas palmitoleic acid was the most potent growth inhibitor against RN4220 with rapid membrane depolarisation and disruption of all major branches of macromolecular synthesis on the bacterial cells. The study suggested that the absence of teichoic acids, a reduction in the level of D-alanine modification, and the absence of major surface protein IsdA could all increase the sensitivity of *S aureus* to fatty acids. There are also studies of multi-drug resistance ATP-binding cassette transporters that are associated with the multi-drug resistance mechanism of cancer cells. In our preliminary study using an ATP-binding cassette p-glycoprotein overexpressed human cancer cell line HepG2, incubation of oleic acid (32 μg/mL) enhanced the rhodamine 123 (a specific substrate to p-glycoprotein transporter) uptake of cells (data not shown). This finding suggests that use of oleic acid may be extended to the development of multi-drug resistance cancer adjuvant therapy.

For *in vivo* studies, sodium linoleate and sodium oleate at a suboptimal dosage could significantly inhibit the growth of RN4220-pUL5054 and ATCC25923, although the effect was inferior to that of vancomycin. This may mainly be due to the possible side effect (acute respiratory distress syndrome) of the unsaturated fatty acid that may worsen in bacteria-induced pneumonia. The dosage of both fatty acids could not be further increased to examine a potentially stronger antibacterial activity in animals. In this regard, the mice pneumonia model may not be an ideal model to evaluate the antibacterial effects of unsaturated fatty acids. We aim to confirm the antibacterial activity of linoleic and oleic acids using skin and wound infection models and examine their potential therapeutic effects in skin and soft tissue infections with MRSA in future studies.

Conclusions

Two active ingredients, namely linoleic and oleic acids, were identified from F18 of PO with synergistic antibacterial activity with erythromycin against MRSA RN4220/pUL5054 *in vitro* and *in vivo*. The effect is likely mediated through inhibition of the efflux pumps of bacteria cells.

Acknowledgement

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Functional profiling and strategic antimicrobial manipulation of a universal nutrition-sensing network to regulate microbial virulence, antibiotic tolerance, and stress protection

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K E Y   M E S S A G E S

1. Nutrient limitation can trigger time-dependent onset of antibiotic tolerance phenotypes.

2. Starvation for 24 hours produced a large population of antibiotic persisters that reverted slowly to a drug-sensitive mode.

3. Inactivation of specific tolerance mechanisms has the potential to reduce the ability of an organism to develop antibiotic resistance.

4. Supplementation of small molecules such as amino acids is highly effective in abolishing antibiotic tolerance phenotypes.

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Introduction

Bacterial stress responses have been postulated to play a role in the onset of recurrent, latent, and biofilm-associated infections, as well as the development of antibiotic resistance.[1,2] Bacteria respond to sub-inhibitory concentrations of antibiotics by modulating their metabolic and gene expression profiles.[3] Production of observable resistance phenotypes such as those attributable to mutations or acquisition of specific resistance determinants may represent the secondary effects of genetic, structural, physiological, and morphological alterations associated with stress responses. This must be taken into account when defining the molecular basis of clinical resistance. It is therefore important to determine whether bacterial stress defence mechanisms that are induced by antibiotics or other adverse factors play a role in the activation of key resistance mechanisms.

This study aimed to identify the determinants of the nutrition-dependent stress response network, their potential role in resistance formation, and the feasibility of suppressing bacterial survival fitness by manipulating the stress response.

Methods

Analyses of time-dependent starvation-induced phenotypes and recovery characteristics

The *Escherichia coli* K-12 strain BW25113 was grown in lysogeny broth to the exponential phase and re-suspended in 3-(N-morpholino)-propanesulfonic acid (MOPS) base. Induction time was set at 10, 30, and 90 minutes, and 24 hours, followed by treatment with three antibiotics (ampicillin, ofloxacin, and gentamicin at 25x minimum inhibitory concentration) for 3 and 48 hours, and assessment of the size of the surviving population as described previously.[4] A parallel persister assay was performed in which organisms were recovered from 24 hours of starvation, re-suspended in Rich Defined Medium (RDM) [Teknova, Holister, CA, USA] and incubated at 37°C for 15 minutes, followed by drug treatment for 3 hours and determination of the size of the persister subpopulation. A gene knockout study was performed to investigate the relative role of selected determinants in regulation of the sustainable tolerance and persistence phenotypes observable during prolonged starvation and the subsequent resuscitation experiments, respectively. All knockout strains tested were obtained from the Keio collection.[5]

Identification of key cellular components responsible for formation of antibiotic tolerance

The following bacterial populations were subjected to analysis using a transcriptome sequencing approach: (1) log-phase populations, (2), stationary-phase population, (3) populations subjected to spontaneous starvation, (4) populations subjected to prolonged starvation stress, (5) populations subjected to combined starvation and antibiotic stress by adding 100 µg/mL ampicillin for 30 minutes, and (6) antibiotic persisters that were produced

by re-suspending bacterial populations with 24-hour starvation in lysogeny broth, followed by treatment with ampicillin at 100 µg/mL to eradicate non-persisters. All transcriptome sequencing experiments were performed by BGI-Hong Kong (http://www.genomics.cn).

Assessment of the putative role of stress defence mechanisms in the development of antibiotic resistance

A resistance formation assay was used to determine the relative potential of selected stress response mutants to develop drug resistance upon antibiotic induction and selection. Briefly, mutation prevention concentration of the test strains was determined by spreading 1×10^9 cells on lysogeny broth agar plates containing ciprofloxacin at a concentration of 0 to 32 µg/mL followed by incubation at 37°C for up to 72 hours. Viable counts on each plate were recorded. Mutation prevention concentration was defined as the lowest antibiotic concentration at which no colonies were observed.

Strategic development of a central approach to control microbial viability

The combined effect of nutrient limitation and deletion of key tolerance determinants on bacterial tolerance induction and survival against environmental stress was tested using a phenotype array approach (Biolog). Briefly, log-phase populations of both wild type and mutant strains were switched to a MOPS-based buffer and aliquoted into a phenotype array containing a range of compounds (P1-P8 of Biolog Phenotype Array), followed by treatment with 100 µg/mL ampicillin. Assessment of bacterial survival at 3 and 48 hours was by inoculation of 1 µL of the test population from each well of the phenotype array onto a lysogeny broth plate.

Results

Phenotypic and molecular characteristics of starvation-induced antibiotic tolerance

The time-dependent induction effects of nutrient limitation on phenotypic antibiotic tolerance were studied by reconstituting RDM-grown log phase cells in MOPS base, followed by exposure of the test organisms to starvation stress for up to 24 hours and assessment of the relationship between the exposure time and drug tolerance phenotypes. The size of the emerging tolerant population increased proportionately with the length of starvation period (Fig 1). Induction for as short as 10 minutes (the shortest induction time testable due to the requirement for medium switching) produced antibiotic tolerance that generally could not be sustained for 48 hours. In comparison, a significant level of sustainable tolerance was consistently produced if the organisms were starved for 24 hours.

Upon reconstitution in RDM, each of the test populations that had been subjected to starvation for ≥30 minutes was found to contain subpopulation persisters that exhibited at least 3-hours tolerance to the three test drugs (Fig 1). Nonetheless, no persisters survived 48 hours of drug treatment. The population size of gentamicin persisters was consistently smaller than that for ampicillin and ofloxacin in all cases. Resembling the tolerance induction characteristics, the potential to develop persisters was also time-dependent, with the level inducible by brief starvation considerably smaller than that following an extended starvation period (90 minutes and 24 hours).

A gene knockout study was performed to investigate the relative role of selected stress response genes in regulation of the sustainable tolerance and persistence phenotypes. The genetic determinants tested were found to play common, differential, or drug-specific roles in starvation-mediated responses, producing a variety of defects (Fig 2). Of particular interest, the *ubiF* and *sucB* loci, both energy production genes implicated in persister formation, were important for such a process. Nonetheless, sustainable tolerance to the test drugs was not affected in the Δ*ubiF* mutant, indicating that the product of this gene was highly specific in mediating persister formation. On the contrary, deletion of *sucB* resulted in defective production of all phenotypes except sustainable tolerance to ofloxacin, suggesting that energy production is required for maintaining the tolerance mode upon nutrient replenishment but not necessary for sustaining such phenotype during starvation.

Among the mutants that displayed an altered phenotypic pattern in the tolerance and persister assay, a drastic difference in the levels of sustainable tolerance and subpopulation persisters was often observed. This indicates that cellular mechanisms responsible for prolonged bacterial survival against antibiotic stress during nutrition starvation were not identical to those required for maintaining the tolerance phenotypes beyond the starvation phase. The fact that prolonged starvation stress enabled various mutants to develop sustainable tolerance to gentamicin, a phenotype not inducible during brief starvation, also confirmed that progressive physiological changes occurred during prolonged starvation.

Identification of key tolerance determinants by transcriptome sequencing analyses

Transcriptome sequencing was performed to probe the molecular basis of physiological changes observed under short- and long-term starvation. A significant discrepancy was observed between populations recovered under different
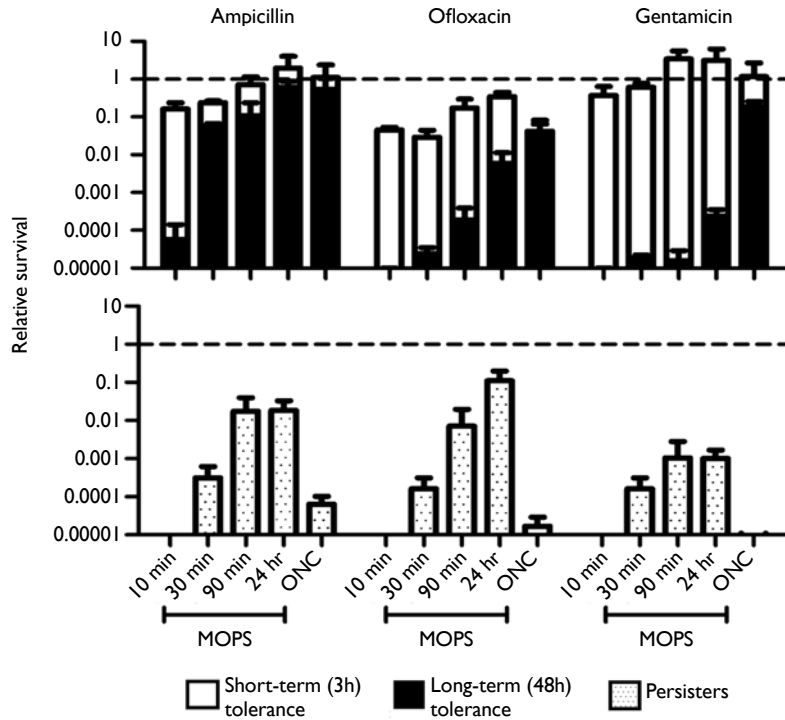
FIG 1. Progressive development of antibiotic persisters within starvation-induced antibiotic-tolerant populations. Exponentially growing *Escherichia coli* BW25113 populations (at 10⁷/mL) in Rich Defined Medium (RDM) were washed and reconstituted in 3-(N-morpholino)-propanesulfonic acid (MOPS) base. At indicated times, aliquots were assayed for tolerance to ampicillin, ofloxacin, and gentamicin as described previously.[4] The relative abundance of persisters at each time point was also determined by reconstituting the MOPS-starved cells in RDM for 15 minutes, followed by 3-hour antibiotic challenge and assessment of the survival rate. An overnight culture (ONC) was included in both assays as control. Abundance of persisters in ONC was determined by 100-fold dilution in RDM, followed by 15 minutes incubation and antibiotic challenge.
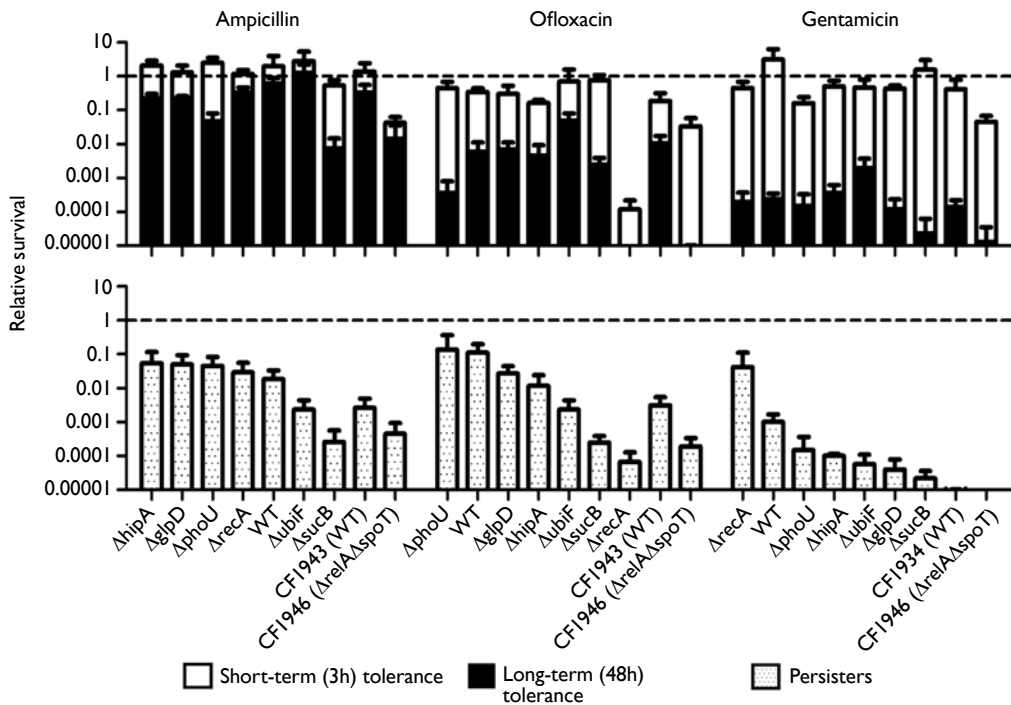
FIG 2. Gene knockout analysis of the relative role of putative persister genes in starvation-induced persister formation. Rich Defined Medium–grown log-phase populations with indicated isogenic gene deletions (arranged in descending order by their relative persister abundance except for the ppGpp+ strain and its corresponding ppGpp0 mutant) were subjected to starvation in 3-(N-morpholino)-propanesulfonic acid base for 24 hours and subsequently analysed for tolerance (to ampicillin, ofloxacin, and gentamicin) and persister development.

TABLE. Results of a representative phenotype array (Biolog) designed to test the effect of specific carbon or amino acid source in abolishing 3-(N-morpholino)-propanesulfonic acid (MOPS)–induced ampicillin tolerance. Ω and Δ denote positive detection of viable organisms in the wild type and Δ*acrA* strain, respectively, after 48 hours challenge by 100 µg/mL ampicillin, ie tolerance remains inducible by MOPS.

	1	2	3	4	5	6
A	Negative control: Ω	L-arabinose: Ω	N-acetyl-D- glucosamine	D-saccharic acid	Succinic acid	D-galactose
B	D-serine	D-sorbitol	Glycerol: Ω	L-fucose	D-glucuronic acid: Ω	D-gluconic acid
C	D-glucose-6-phosphate	D-galactonic acid-γ-lactone	D,L-malic acid	D-ribose	Tween 20	L-rhamnose: Ω
D	L-asparagine	D-aspartic acid: Ω Δ	D-glucosaminic acid	1,2-propanediol: Ω Δ	Tween 40	α-keto-glutaric acid
E	L-glutamine	m-tartaric acid	D-glucose-1- phosphate	D-fructose-6- phosphate	Tween 80	α-hydroxy glutaric acid-γ- lactone
F	Glycyl-L-aspartic acid	Citric acid	m-inositol: Ω	D-threonine	Fumaric acid: Ω	Bromo succinic acid
G	Glycyl-L- glutamic acid	Tricarballylic acid: Ω	L-serine	L-threonine: Ω	L-alanine	L-alanyl-glycine: Ω
H	Glycyl-L-proline	p-hydroxy phenyl acetic acid: Ω	m-hydroxy phenyl acetic acid: Ω	Tyramine	D-psicose	L-lyxose: Ω Δ

test conditions. A large number of genes was up-regulated in populations subjected to spontaneous and prolonged starvation, when a log-phase population was used as control. The discrepancy between the expression profile of stationary phase cells and those subjected to starvation stress revealed that nutrition limitation was not the only factor that induced development of phenotypic tolerance in stationary-phase populations. Antibiotics induced further stress responses in bacterial populations undergoing a starvation process, and the antibiotic tolerance phenotypes observed during starvation may be partially attributed to physiological changes triggered by the antibiotic itself. The transcription profile of persisters differed from that of the starving population, indicating that persisters comprised only a fraction of the original tolerant population detectable during starvation.

Transcriptome studies were performed to identify genes that are significantly up-regulated during prolonged starvation. When a bacterial population subjected to 24 hours starvation was compared with a log-phase population, >1700 genes were found to be up-regulated. Genes that were exclusively expressed during starvation and those for which the transcription level was most significantly up-regulated included the TetR/AcrA transcriptional activator, multiple stress resistance protein BhsA, and DNA repair protein RadC. A huge amount of transcriptome sequencing data are being analysed to shortlist genetic components for future studies to assess their potential as drug targets to reduce survival fitness of antibiotic tolerant and resistant organisms.

Assessment of the putative role of stress defence mechanisms in the development of antibiotic resistance

To determine whether strains that are compromised in their ability to develop or maintain antibiotic tolerance phenotypes are also less able to develop into resistant mutants, the mutation prevention concentration of selected gene knockout mutants was determined (Δ*sodA*, Δ*arcA*, Δ*dnaK*, Δ*dps*, Δ*proP*, Δ*rcsC*). The wild type and all test strains except Δ*arcA* exhibited a mutation prevention concentration of 0.25 µg/mL. For Δ*arcA*, no growth or emergence of mutants was detectable at any test concentration (the lowest being 0.06 µg/mL), indicating that failure to produce the *arcA* gene product not only leads to a lower tolerance level, but also undermines the ability of the organism to undergo mutational changes and develop resistance phenotypes. The Δ*dnaK* mutant was also found to produce a substantially smaller number of colonies on the plates containing 0.06 and 0.12 µg/mL, suggesting that a lack of *dnaK* gene product also affected development of both tolerance and resistance phenotypes.

Strategic development of a central approach to control microbial viability

A phenotype array approach was used to test the combined effects of nutrient supplementation and gene knockout on tolerance formation. A total of 760 compounds including amino acids and various carbon and nitrogen sources were tested for their ability to abolish MOPS-induced tolerance to ampicillin in a wild type strain and the *acrA* deletion mutant. A substantial number of compounds were able to abolish antibiotic tolerance. For the wild type strain, 210 (28%) of the 760 compounds tested conferred the ability of ampicillin to eradicate the entire test population in a MOPS-based environment that is supposed to confer prolonged tolerance to this drug. For the Δ*acrA* strain, as many as 363 (48%) compounds could cause abolition of ampicillin tolerance (Table), indicating that nutrient supplementation can enhance the effects of knockout of specific genes in suppressing the

7	8	9	10	11	12
L-aspartic acid	L-proline	D-alanine	D-trehalose	D-mannose	Dulcitol
D,L-α-glycerol-phosphate	D-xylose	L-lactic acid: Ω	Formic acid: Ω	D-mannitol	L-glutamic acid
D-fructose: Ω	Acetic acid	α-D-glucose	Maltose	D-melibiose	Thymidine
α-keto-butyric acid	α-methyl-D- galactoside	α-D-lactose: Ω	Lactulose	Sucrose: Ω	Uridine
α-hydroxy butyric acid	β-methyl-D- glucoside: Ω	Adonitol	Maltotriose	2-deoxy adenosine	Adenosine
Propionic acid	Mucic acid	Glycolic acid: Ω	Glyoxylic acid	D-cellobiose	Inosine
Acetoacetic acid	N-acetyl-β–D- mannosamine	Mono methyl succinate	Methyl pyruvate	D-malic acid: Ω	L-malic acid
Glucuronamide	Pyruvic acid	L-galactonic acid-γ-Lactone	D-galacturonic acid	Phenylethyl-amine	2-aminoethanol

ability of the host organism to develop and maintain antibiotic tolerance.

Discussion

This study describes a sequential developmental pattern of antibiotic tolerance in bacteria subjected to nutrient limitation, in which the physiological responses elicited are highly dependent on the duration of stress exposure. Tolerant populations generated by prolonged starvation also harboured a subpopulation of persisters that reverted only slowly into an actively growing, drug-sensitive mode upon replenishment of nutrients. The size of this persister population varies according to the length of the preceding starvation period. This indicates that starvation stress elicits long-lasting protective mechanisms that confer resistance to multiple stresses. This is consistent with our previous findings that starvation-induced stress tolerance was not solely due to physiological dormancy,[4] which would not increase strength of tolerance as starvation conditions persist. Results of our gene expression profiling and gene knockout experiments confirmed the presence of independent yet overlapping genetic pathways that regulate the drug specificity, sustainability, and reversibility of antibiotic tolerance and that active defence functions such as efflux and production of chaperone proteins constitute the key tolerance mechanisms.

Apart from being potential targets of drugs designed to eradicate tolerant or persistent bacterial populations, cellular components responsible for producing antibiotic tolerance phenotypes may also be good targets for the control of resistant organisms. The intricate relationship between tolerance and resistance needs further investigation. Our findings suggest that suppressing stress responses can reduce the chance of resistance formation. Preliminary tests have been performed to examine the feasibility of using small nutrient molecules to interfere with the ability of bacteria to withstand environmental stresses. Amino acids and various carbohydrates and nitrogen sources are highly potent in abolishing tolerance phenotypes. Thus, agents that suppress tolerance development may reduce bacterial survival fitness, thereby enhancing the effects of conventional drugs.

Conclusion

Our findings may facilitate the development of new generation antimicrobial drugs through widespread interference and suppression of bacterial stress responses. This new antimicrobial approach with both natural and specially designed agents may be used to suppress multiple cellular pathways.

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