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Research Fund for the Control of Infectious Diseases

Research Dissemination Reports

Commissioned studies by the Chinese University of Hong Kong

控制傳染病研究基金

研究成果報告

香港中文大學委任研究

Infection Control
感染控制

Respiratory Infectious Diseases
呼吸道感染疾病

Viral Hepatitis
病毒性肝炎

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Research Fund for the Control of Infectious Diseases

Research Dissemination Reports

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Viral Hepatitis

After the outbreak of severe acute respiratory syndrome (SARS) in Hong Kong in 2003, the *Research Fund for the Control of Infectious Diseases* (RFCID) was established to encourage, facilitate and support research on the prevention, treatment and control of infectious diseases, in particular emerging infectious diseases, so as to formulate policies. Over the course of 5 years, researchers in the Chinese University of Hong Kong completed a portfolio of basic, epidemiological, public health and clinical research on a diverse range of potentially emerging and re-emerging infectious diseases, including SARS, influenza, viral hepatitis, and gastrointestinal pathogens. Evidence-based knowledge generated from these projects has helped in health policy formulation and health care services delivery. In this issue, a representative selection from the portfolio is presented. Three projects are highlighted owing to their contribution to knowledge on emerging and re-emerging pathogens and their impact on patient care.

With any outbreak of a novel pathogen, early identification and isolation of infected individuals is important in the effective control of an epidemic. Following the outbreak of SARS, Lo[1] developed a novel plasma/serum RNA test for SARS-coronavirus (CoV) infection. Using this assay, plasma SARS-CoV RNA concentrations in ribavirin-treated patients who received early hydrocortisone therapy were compared with those who received placebo. SARS-CoV RNA was detected 3 to 4 days after fever onset, and its concentration peaked in the first week and rapidly declined to become undetectable after 20 days. Plasma SARS-CoV RNA concentrations in the second and third weeks of illness were significantly higher in patients who received initial hydrocortisone treatment compared with those who received placebo. Serum SARS-CoV concentration has prognostic implications and serial assessment is useful for the monitoring of patient progress.

The long-term health consequences of infection by novel pathogens are unknown. Hui et al[2] studied the long-term sequelae (ie pulmonary function, exercise capacity and quality of life) of SARS-CoV infection in a prospective longitudinal follow-up study of 123 patients with SARS discharged from a single hospital. About 25% of the survivors had impaired lung diffusion capacity and/or abnormal chest radiographs 12 months after illness onset. In addition, exercise capacity and health status of SARS survivors were significantly lower than in age-matched normal controls. Thus, SARS-CoV infection caused long-lasting physical and psychological impairment in a significant proportion of survivors.

Severe seasonal influenza is responsible for about 15 to 50 hospital admissions per 10 000 of the elderly population in Hong Kong. Those affected may suffer complications including pneumonia, bronchitis, exacerbations of chronic pulmonary diseases, heart attacks and strokes. Mortality among hospitalised patients can approach 30%. Few clinical studies on immunopathogenesis have been performed on patients with severe human influenza infections. Lee[3] examined the role of cytokines and chemokines in severe and complicated influenza H1N1 infection in 39 adult patients. The concentrations of many cytokines (including IL-6, IL-8, IP-10, MIG and MCP-1) were elevated in the acute phase as compared to the convalescent phase. This hypercytokinaemia usually occurred in patients of advanced age, with major co-morbidities, and with cardio/respiratory complications. Early, effective viral suppression may result in attenuation of those harmful cytokine responses giving rise to such complications, and further studies are warranted.

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

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Risks posed by the use of oxygen therapy and non-invasive positive pressure ventilation: a pilot study

Key Messages

1. Substantial exposure to exhaled air occurs within a 0.5 m radius of patients receiving non-invasive positive pressure ventilation and a 0.4 m radius of those receiving oxygen therapy.
2. Health care workers should take protective precautions when managing patients with community-acquired pneumonia complicated by respiratory failure.

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Introduction

Community-acquired pneumonia (CAP) is a common disease with high morbidity and mortality. Patients with CAP may require various forms of respiratory support, including supplemental oxygen delivered via nasal cannulae or face masks, non-invasive positive pressure ventilation (NPPV), and invasive mechanical ventilation. There is a strong association between ventilation, air movements in buildings, and the transmission of infectious diseases such as measles, tuberculosis, chickenpox, influenza, smallpox, and SARS.[1] In patients with viral pneumonia, there is a potential risk that the respiratory therapy may generate and disperse infective aerosols, resulting in a super-spreading event. The use of a nebuliser in an overcrowded medical ward with inadequate ventilation is thought to have caused a nosocomial SARS outbreak in our hospital in 2003.[2-4]

Respiratory failure is the major complication in patients with influenza A/H5N1 infection, and many such patients progress rapidly to acute respiratory distress syndrome and multi-organ failure.[5] The US pandemic influenza plan recommends health care workers (HCW) to take precautions against airborne transmission of infection when managing patients with pandemic influenza of increased transmissibility and during procedures that may generate small aerosol particles of respiratory secretions.[6] As part of our preparation for pandemic influenza, we studied the dispersion distances of air particles during application of NPPV and oxygen therapy via standard masks attached to a high fidelity human patient simulator (HPS).

Aims and objectives

Viruses such as influenza may be spread by airborne particles and droplets. It is not known how exhaled air and particles are dispersed during the application of NPPV and oxygen therapy in clinical settings. There is no reliable marker that can be safely introduced into patients to enable such a study. Using laser visualisation techniques on a high fidelity HPS, we studied air and particle dispersion distances during the use of NPPV and oxygen therapy, via a simple oxygen mask, to the HPS.

Methods

This study was undertaken by a multidisciplinary team of investigators consisting of physicians, intensivists, anaesthetists, architects, and an aeronautical engineer. It was conducted in a quiet laboratory room measuring 7.1×8.5 m, with a height of 2.7 m. The ventilation was temporarily suspended during the experiment to avoid potentially confounding environmental factors, such as air currents.

Non-invasive positive pressure ventilation and the lung model

We studied the mask interface leakage and deliberate leakage from the exhaust holes of an oronasal mask (Ultra Mirage Medium, ResMed, Sydney, Australia) firmly attached to an HPS (HPS 6.1, Medical Education Technologies, Sarasota [FL], US). The HPS is a realistic representation of human respiration. It contains a realistic airway and a lung model that performs gas exchange, ie it removes oxygen and adds carbon dioxide to the system. The lung compliance and airway

resistance also respond in a realistic manner. The HPS also produces an airflow pattern close to the in vivo human pattern, and has been used in previous studies to simulate human respiration.[7-11]

A bi-level positive airway pressure device (ResMed VPAP III ST, Sydney, Australia) was used via an oronasal mask. The inspiratory positive airway pressure (IPAP) was initially set at 10 cm H_2O, and gradually increased to 18 cm H_2O. The expiratory positive airway pressure (EPAP) was maintained at 4 cm H_2O throughout this study.[12]

Simple oxygen mask and the lung model

We studied the air particle leakage from the side vents of a simple oxygen mask (HS-3031, Hsiner, Taichung Hsien, Taiwan) applied to the HPS. Oxygen was delivered to the HPS via the simple mask at 4 L/min initially, then gradually increased to 6, 8 and 10 L/min.[13]

The lung compliance and oxygen consumption of the HPS was set to 35 mL/cm H_2O and 350 mL/min, respectively. Tidal volumes and respiratory rates were regulated so that a respiratory exchange ratio of 0.8 was maintained. This gave a tidal volume of 500 mL at a rate of 14 breaths/min, which represented a patient with a mild lung injury.[14,15] While the HPS was breathing oxygen at 6 L/min with the simple oxygen mask, coughing was produced by a short burst (2 sec, 400 L/min) of air (marked by smoke) generated by a jet ventilator (Monsoon, Acutronic Medical Systems, Baar, Switzerland) connected to the proximal trachea. This represented coughing efforts in patients with mild lung injuries.[16]

Flow visualisation

Visualisation of airflow around the interface mask was facilitated by marking air with smoke particles produced by a M-6000 smoke generator (N19, DS Electronics, Sydney, Australia).[12,13] The oil-based smoke particles (<1 μm in diameter) are known to follow the airflow pattern precisely with negligible slip.[17] The smoke was introduced to the right main bronchus of the HPS continuously. It mixed with alveolar gas, and was then exhaled through the airway. Sections of the leakage jet plume were then revealed by a thin laser light sheet created by an Nd:YVO_4 Q switched, frequency-doubled laser (OEM T20-BL 10-106Q, Spectra-Physic, USA).[12,13]

The experiments were recorded with a digital video system (3CCD, 48X zoom, 30 Hz). The laser light sheet (Green, 527 nm wavelength, TEM_{00} mode) was adjusted to encompass the largest cross-section of the entire leakage jet plume. The light sheet was initially positioned in the median sagittal plane of the HPS then shifted to the paramedian planes. This enabled investigation of the regions directly above and lateral to the mask and the patient.[12,13]

Image analysis

We estimated the normal smoke concentration of the oxygen mask leakage jet plume from the light scattered by the smoke particles.[8] This extended the laser flow visualisation and provided a useful quantitative method for understanding the range and shape of the leakage jet plumes. This technique was previously developed for turbulent two-phase, air-particle flows and has been proven as reliable as isokinetic sampling[18] or particle image velocimetry.[19]

The laser light sheet illuminated the smoke particles around the NPPV mask and the oxygen mask. The laser light scattered by the smoke particles was then collected by the video camera. In a small space, the number of smoke particles within the space (or the particle concentration) is proportional to the total scattered light intensity in the corresponding area, because the smoke particles were all the same size, or monodisperse.[17]

Image capture and frame extraction

The motion of several breathing cycles at a given oxygen flow rate setting was recorded in a computer, and individual frames extracted as grey-scale bitmaps for intensity analysis. Frames were extracted at times corresponding to the beginning of inspiration (at a given oxygen flow rate) to generate an ensemble average for the corresponding instant of the respiratory cycle. The largest spread of contours from the mask was chosen and this was found to be at approximately the mid time of the respiratory cycle.[12,13]

Results

During the application of NPPV, a jet plume of air leaked through the mask exhaust holes to a radial distance of 0.25 m from the mask when IPAP was 10 cmH_2O, with some leakage from the nasal bridge. The leakage plume exposure probability was highest about 60-80 mm lateral to the median sagittal plane of the HPS. Without nasal bridge leakage, the jet plume from the exhaust holes increased to a 0.40 m radius from the mask, whereas exposure probability was highest about 0.28 m above the patient. When IPAP was increased to 18 cm H_2O, the vertical plume extended to 0.45 m above the patient with some horizontal spreading along the ceiling.[12]

During delivery of oxygen via a simple mask at 4, 6, 8 and 10 L/min, a jet plume of air leaked through the side vents of the mask to a lateral distance of 0.2, 0.22, 0.3, and 0.4 m from the sagittal plane, respectively. Coughing could extend the dispersion distance beyond 0.4 m.[13]

Discussion

Air marked with smoke particles can be emitted through deliberate mask leakage to a radial distance of approximately 0.25 m from an oronasal mask during application of NPPV. The leakage jet plume was most significant about 60-80 mm lateral to the median sagittal plane. Despite the use of a reasonably well-fitted mask, leakage was still detected at the nasal bridge. With elimination of this nasal bridge

leakage, the jet plume radial distance from the mask increased to 0.4 m, with exposure probability highest about 0.28 m above the patient and the mask. When IPAP was increased to 14 cm H_2O and then 18 cm H_2O, the vertical plume extended to 0.42 m and 0.45 m respectively above the patient, with some horizontal spreading along the ceiling.[12] These findings have important clinical implications for HCW who often nurse their patients at a close distance, especially during NPPV support for respiratory failure, at a stage when viral loads may reach peak levels. Our study emphasises the importance of medical ward design for ensuring a ventilated, aerodynamic space and the need for an architectural aerodynamics approach to minimise the risk of nosocomial infection. Air-conditioning or extraction systems need to target the circular region above the mask rather than the actual mask level.

This study simulates a worst-case scenario in order to demonstrate the maximum distribution of exhaled air. Using laser smoke visualisation methods, we showed that exhaled air dispersed at maximal distances of 0.2, 0.22, 0.3, and 0.4 m (for contours above 20%) lateral to the median sagittal line of the HPS when oxygen was delivered via a simple mask at 4, 6, 8 and 10 L/min respectively. Within these dispersal distances from the mask, the chance of exposure to the patient's exhaled air is greater than 20%. Thus within dispersal distances of 0.16, 0.17, 0.25, and 0.35 m, there was at least a 60% chance of exposure to the exhaled air at oxygen flows of 4, 6, 8, and 10 L/min respectively. Coughing increased the air dispersion distance from 0.17 m to 0.2 m when the HPS was receiving 6 L/min of oxygen with at least a 60% chance of exposure within the distance.[13] These findings have important clinical implications for HCW who often manage patients with CAP at a close distance. A case control study involving 124 medical wards in 26 hospitals in Guangzhou and Hong Kong has identified six independent risk factors for super-spreading nosocomial outbreaks of SARS. They are the minimum distance between beds <1 m, performance of resuscitation, staff working while experiencing symptoms, and SARS patients requiring oxygen therapy or NPPV. The availability of washing or changing facilities for staff was a protective factor.[20] It is important to provide adequate respiratory protection for HCW, in addition to applying standard contact and droplet precautions, in order to prevent nosocomial infections.

This study was limited by the use of smoke particles as markers for exhaled air. The inertia and weight of larger droplets would certainly cause them to have less horizontal dispersion. Nevertheless, the evaporation of water content in some droplets during NPPV or oxygen therapy may produce droplet nuclei suspended in air, whereas the larger droplets will fall to the ground in a trajectory pathway. As the smoke particles in this study mark the continuous air phase, our data contours are referring to exhaled air. Our results represent the upper boundary of estimates for the dispersion of droplets that would be expected to follow a shorter trajectory than the air jet due to gravitational effects, but not fully reflect the risk of droplet transmission. In addition, ventilation was switched off during the experiments in order to reveal the maximum distribution of exhaled air without interference by external factors. Further work is needed to assess the interaction between ward ventilation and the dispersion distances during different modes of therapy for respiratory failure. We were unable to capture the maximum dispersion distance during coughing due to limitations of the equipment. A higher speed camera with a wider laser beam is required to detect the full range of dispersion distances during manoeuvres such as coughing and sneezing.[12,13]

Conclusions

Substantial exposure to exhaled air occurs within a 0.5 m radius of patients receiving NPPV and a 0.4 m radius of those receiving oxygen therapy. Higher ventilator pressures result in a wider distribution of exhaled air. Coughing can extend the dispersion distance further.

Health care workers should be aware of the potential risks of viral transmission during application of NPPV and oxygen therapy for patients with CAP. It is advisable to follow the World Health Organization recommendation that precautions for airborne transmission be adopted in health care facilities, including placing patients with suspected and confirmed H5N1 influenza in isolation rooms with at least 12 air exchanges/hr during aerosol-generating procedures. Further studies are needed to examine the dispersion of exhaled air during common respiratory therapies in relation to the air exchange rate and airflow patterns in medical wards, and its role in the control of airborne virus infection.

Acknowledgements

This project forms part of a series of studies commissioned by the Food and Health Bureau of the Hong Kong SAR Government and was funded by the Research Fund for the Control of Infectious Diseases. These results have been reported in the following publications:

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Immunogenetic studies in SARS: developing a clinical prognostic profile for severe diseases

Key Messages

1. No major gene was found to influence the course and outcome of illness secondary to SARS-CoV infection.
2. Phenotypic variation in IP-10 expression was not caused by any of the genetic factors investigated in this study.
3. Phenotypic measurements, instead of genetic markers, may be useful in future clinical applications.

Introduction

The 2003 outbreak of SARS in Hong Kong greatly affected the health care system. More than 80% of patients recovered while the remainder suffered a severe disease leading to respiratory failure and admission to the intensive care unit. The course of the disease and outcome were markedly heterogeneous and we hypothesised that these were partly determined by differences in the intensity of host reaction toward the infection.

Our previous investigation of the 1997 avian influenza A (H5N1) outbreak showed that patients who died of the disease had lymphoid depletion associated with marked elevation of circulating concentrations of cytokines, including interleukin-6, interleukin-2 receptor and interferon-gamma. There are many similar features between influenza H5N1 infection and SARS. It is likely that hypercytokinaemia together with associated systemic and local reactions play a key role in the lung damage and determine the disease outcome in patients with SARS.

There is no good predictor of disease outcome after SARS infection. Old age, higher neutrophil count, and serum lactate dehydrogenease were the only markers associated with subsequent admission to the intensive care unit. However, by themselves these are only certainly surrogate markers of disease intensity and immunocompetence. The underlying determinants remain unknown. The ability to predict the disease course may strongly influence the choice of treatment regimen, especially if severe disease could be anticipated early after admission to hospital.

IP-10 expression levels after SARS-CoV infection might be associated with disease prognosis.[1] It was uncertain whether such phenotypic variation was caused by a genetic difference (variation) among individuals or by environmental factors (such as the extent of virus exposure or other host factors). We studied the immune response at the genetic level in SARS patients. Genomic polymorphisms of inflammatory mediators accounting for variations in the intensity of an individual's immune reaction against a pathogen and circulating cytokines levels were studied as prognostic markers in patients who developed SARS infection. We hypothesised that patients with a particular high-risk genotype might have a more intense inflammatory response.

Aims and objectives

1. To characterise the inflammatory (particularly chemokine) responses in SARS-CoV infection.
2. To evaluate whether the differences in disease outcome between patients were related to genetic factors.

Methods

Study design

This was a genetic association study. A total of 677 SARS patients (including 500 controls) were studied to determine the genetic polymorphisms between

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groups of patients. In a subgroup analysis, SARS patients with adverse outcomes were compared with SARS patients who recovered (controls).

Genotyping of candidate genes

Genotypes of selected candidate genes were determined from DNA extracted from blood samples using a commercial DNA extraction kit. Representative variations (commonly single nucleotide polymorphisms [SNPs]) were genotyped by an established protocol. The frequencies of genotypes and alleles of each SNP were compared between SARS patients who had adverse outcomes and patients who recovered. As the sample size of both groups was limited, we also compared the frequencies of the alleles in the case group with those found in the population. We also estimated the population allele frequencies of each SNP.

Genotyping results were determined under stringent quality control procedures that included repeat genotyping of 1-2% of samples determined for each genotype, false positive polymerase chain reaction results and inclusion of standard samples in all batches of reactions.

Statistical analysis

Hardy-Weinberg equilibrium of alleles of individual genes was assessed by exact tests using a population genetics software (GENEPOP). Comparison of genotype frequencies between cases and controls were analysed by Chi squared tests. Univariant analysis was carried out to identify genotypes that were associated with adverse outcomes. The correlation between genotypes and inflammatory response/disease outcome was analysed by linear/logistic regression.

Results

Genetic variations in both forms of ACE genes (ACE1 and ACE2) were not a risk factor for severe disease prognosis after SARS infection. Genetic polymorphisms in the L-SIGN gene, another putative receptor for the virus, were also not associated with prognosis or disease susceptibility. Notably, genetic factors affecting both chemokine and cytokine genes were not associated with prognosis.

Discussion

Chemokine expression levels (particularly IP-10) was an important factor associated with disease prognosis, but the cause of such phenotypic variation was not certain. It may be due to a genetic difference between individuals or differences in environmental factors, such as extent of viral exposure, concurrent medical conditions, or other factors. This study explored a number of candidate genes considered important in the pathogenesis of infection and showed that differences between them were not related to differences in prognosis after SARS infection.

Variation in levels of serum inflammatory mediators reflects phenotypic differences in host inflammatory reactions during an infection. The intensity of immune response might also be genetically determined. The differences in genetic makeup between individuals are mostly accounted for by single-base differences known as SNPs. Many studies show an association between SNPs and predisposition to adult respiratory distress syndrome (ARDS) and survival after sepsis or other insults.[2,3] In the context of predisposition to ARDS after trauma, the interaction between circulating concentrations of interleukin-1, tumour necrosis factor and plasminogen activator inhibitor–1 and the genotype for plasminogen activator inhibitor–1 (PAI-1) have been studied.[4] In addition to PAI-1, other genetic polymorphisms were also associated with a predisposition to and/or severity and outcomes of ARDS, including angiotensin-converting enzyme,[2] CD14,[5] surfactant protein,[6] and HLA genotypes.[7] The association between alleles of the two ACE genes (ACE and ACE2) and severity of ARDS after SARS infection revealed negative results.[8,9] In addition, a study on novel SNPs identified by resequencing of the ACE2 gene also yielded no association with SARS infection.[10]

Several other immunogenetic studies have been reported in association with SARS infection, two of which suggested such association with HLA genotype. Among 37 Taiwan SARS patients, HLA-B*4601 was associated with both a predisposition to infection as well as the severity of infection.[11] However, the association of this allele could not be replicated in another study in Chinese SARS patients using a larger sample size.[12] In contrast, HLA-B*0703 was found to be a predisposition allele. However, this rare allele is found in about 3% of the general population and could not account for a major predisposition factor for SARS infection.[12] Genetic variation in the L-SIGN gene (CLEC4M) was also not associated with disease severity.[13] It is clear that immunogenetics is an important field in SARS research. However, none of the genes studied so far appear to be important or major determinants of disease outcome.

Conclusions

No major genetic risk factors for disease susceptibility or disease prognosis were determined in this study. Phenotypic determination by assay of chemokine expression levels (eg serum concentration of chemokines) may be important independent risk factors useful in future clinical applications. We should increase awareness of the importance of chemokines in immune responses and review the facilities for measuring them in clinical practice.

Acknowledgements

This project forms part of a series of studies commissioned by the Food and Health Bureau of the Hong Kong SAR Government and was funded by the Research Fund for the Control of Infectious Diseases. The results of this study have been reported in the following publications:

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between angiotensin converting enzyme polymorphism and development of adult respiratory distress syndrome in patients with severe acute respiratory syndrome: a case control study. BMC Infect Dis 2005;5:26.

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SARS diagnosis, monitoring and prognostication by SARS-coronavirus RNA detection

Key Messages

1. We demonstrated the utility of a test for SARS-CoV RNA in serum/plasma in the diagnosis and prognostication of patients and its possible role in serial monitoring of treatment efficacy.
2. An automated viral RNA extraction procedure was found to be less effective than manual extraction.
3. The experience gained in developing the SARS diagnostic test was used to develop rapid methods for genotyping the SARS-CoV.

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Introduction

The 2003 SARS epidemic affected 29 countries around the world.[1,2] The early identification and isolation of infected individuals appeared important for the effective control of such an epidemic.[3] We reported the development of a diagnostic test based on the detection of the SARS-CoV RNA in serum/plasma by real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR)[4,5]; 80% of infected individuals were shown to be positive on the first day of hospital admission with no false-positive results.[4,5] The serum SARS-CoV RNA concentration detected upon admission was predictive of the requirement for subsequent intensive care.[4] Further developments to SARS-CoV RNA detection from serum/plasma may improve our preparedness for future epidemics.[6]

Systematic analysis of SARS-CoV sequence information demonstrated that characteristic viral genotypes predominated at certain periods during the course of the outbreak.[7-10] Furthermore, characterisation of viral sequences is useful for confirming epidemiological associations between infected individuals as suspected from conventional epidemiological investigations.[9-11] In-depth analysis of the available sequence data on SARS-CoV also revealed that the viral isolates could be readily sub-classified into several major genotypes based on nucleotide variations at specific genomic positions.[8,12] Phylogenetic analysis of SARS-CoV sequences revealed a 5-nucleotide motif (GenBank Accession: AY390556; comprising reference nucleotide residues 17,564, 21,721, 22,222, 23,823, and 27,827) that was identified to be most useful for distinguishing the major SARS-CoV genotypes.[8] These major viral genotypes predominated at different periods of the epidemic.[8] Thus, it is evident that viral sequence and molecular epidemiological data provide valuable information to combat infectious diseases. However, direct sequencing of viral isolates from a large number of clinical samples is cumbersome and time consuming. A rapid system for the characterisation and screening of viral genotypes, such as for SARS-CoV, could be useful.

Aims and objectives

Our collaborative group has a long-standing history in the development of novel diagnostic and monitoring tests using plasma and serum. Following the outbreak of SARS, we applied this expertise to the development of a novel plasma/serum RNA test for SARS-CoV infection. Using this assay, we demonstrated, for the first time, the existence of SARS-CoV RNA in the cerebrospinal fluid of a SARS patient with neurological manifestations.

We aimed to (1) enhance our understanding of the scope for applying this plasma/serum RNA-based test, (2) explore ways to further enhance the throughput of the test, and (3) develop assays based on the new technology to rapidly genotype strains of SARS-CoV. Experience gained in developing the serum SARS-CoV RNA test for the diagnosis of SARS may provide valuable insights for the investigation of other emerging infectious diseases.

Methods

Three issues related to SARS-CoV RNA detection were explored:

(1) The role of SARS-CoV RNA detection in plasma/serum for the monitoring of treatment efficacy.
(2) The potential for shortening the turnaround time of the diagnostic assay based on SARS-CoV RNA detection in plasma/serum through automation.
(3) The development of rapid methods for the genotyping of SARS-CoV isolates.

Regarding issue 1, we compared the plasma SARS-CoV RNA concentrations in ribavirin-treated patients who received early hydrocortisone therapy with those who received placebo. Serial plasma SARS-CoV RNA concentrations measured in the setting of a prospective, randomised double-blinded, placebo-controlled trial designed to assess the efficacy of 'early' (before day 7 of illness) hydrocortisone use in previously healthy SARS patients were analysed. SARS-CoV RNA was quantified using a one-step real-time RT-PCR assay targeting the nucleocapsid gene.

Regarding issue 2, we compared the quantitative performance of a manual (Qiagen Viral mini kit) and automated (MagNA Pure LC instrument) protocol for SARS-CoV RNA extraction. We determined the optimal nucleic acid extraction kit to be adopted by the automated system, assessed the possibility of contamination and carry-over by the automated system, and compared the quantitative performance between the optimised automated and manual protocols for the extraction of inactivated SARS-CoV spiked in transport medium and in human serum.

Regarding issue 3, sequence analysis of SARS-CoV isolates revealed that specific genotypes predominated at different periods of the epidemic. This information can be used as a footprint for tracing the epidemiology of infections and monitor viral evolution. However, direct sequence analysis of a large number of clinical samples is cumbersome and time consuming. We aimed to develop a simple and rapid assay for the screening of SARS-coronavirus genotypes based on the use of fluorogenic oligonucleotide probes for allelic discrimination. In a large-scale phylogenetic analysis of SARS-CoV sequences, a 5-nucleotide motif was identified to be most useful for distinguishing the major SARS-CoV genotypes. We focused on the development of allelic discrimination assays for these five characteristic single nucleotide variations (SNV). Each patient's RNA was extracted from viral isolates cultured from clinical specimens using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Eleven microliters of the extracted viral RNA was reverse-transcribed by Superscript III (Invitrogen, Carlsbad [CA], USA) with random hexamer according to manufacturer's instructions. Genotyping of the five SNVs was determined using TaqMan (Applied Biosystems, Foster City, CA, USA) allelic discrimination assays on an ABI Prism 7900HT sequence detection system (Applied Biosystems). Each assay consisted of two allele-specific minor groove binding probes associated with either 6-carboxyfluorescein (FAM) or VIC™ as the fluorescent label. These were to discriminate between the two respective alleles at each SNV locus. One assay was designed for each of the 5 SNVs. The primer and probe sequences were designed using the Primer Express 2.0 software (Applied Biosystems).

Results

In issue 1, among 16 non–intensive-care-unit patients, SARS-CoV RNA was detected in the plasma at day 3-4 after fever onset; viral concentration peaked in the first week, which then rapidly declined in the second week. On days 8, 12, 16 and 20, the cumulative proportion of patients with undetectable virus in plasma was 31%, 69%, 92% and 100%, respectively. Plasma SARS-CoV RNA concentrations in the second and third weeks of illness were significantly higher in patients who received initial hydrocortisone treatment (n=9), as compared to those who received placebo (n=7) (AUC; Mann-Whitney, P=0.023). Their respective median time for SARS-CoV to become undetectable in plasma was 12 (range, 11-20) days and 8 (range, 8-15) days.

In issue 2, the detection sensitivity of the MagNA Pure LC total nucleic acid large volume kit was compared with the MagNA Pure LC total nucleic acid kit. The former kit had superior sensitivity and was therefore adopted for further comparison against the manual extraction method. Samples of viral transport medium spiked with and without inactivated SARS-CoV were arranged in a sequential manner for the assessment of carry-over contamination in the automated system. None of the plain samples revealed positive detection of SARS-CoV RNA, which therefore suggested that the MagNA Pure LC instrument was not prone to carry-over contamination. Median SARS-CoV RNA concentration in transport medium was 3.8-fold higher when extracted by the manual method in contrast to the automated method (Wilcoxon P=0.002). A constant negative bias was also noted in serum SARS-CoV RNA concentrations when extracted by the automated in comparison to the manual protocol (Wilcoxon P=0.002). The detection sensitivities for serum SARS-CoV RNA of both protocols were comparable.

In issue 3, TaqMan allelic discrimination assays for the five SNVs were tested on synthetic templates (Sigma Genosys, Australia) and verified using two viral isolates, CUHK-W1 (GenBank Accession: AY278554) and CUHK-Su10 (GenBank Accession: AY282752). We confirmed that the newly developed allelic discrimination assays were able to differentiate the two viral isolates and genotype each SNV correctly. Following initial development and optimisation, the allelic discrimination assays were used to genotype SARS-CoV in clinical samples. We were able to successfully determine the SARS-CoV genotypes in all the 30 samples studied. The SARS-CoV genotypes isolated from the 30 patients were also confirmed by direct sequencing. The sequencing results were fully concordant

with those based on the allelic discrimination assays at all five SNVs.

Discussion

In young, previously healthy adult SARS patients, SARS-CoV RNA was detected in plasma from day 3-4 after fever onset; peak concentration were detected in the first week, and declined rapidly in the second week. 'Early' hydrocortisone treatment initiated within 7 days of the illness was associated with significantly higher subsequent plasma viral concentrations in the second and third weeks. 'Early' initiation of corticosteroid treatment during the viral replication phase in the first week of illness resulted in delayed viral clearance (thus a higher subsequent plasma viral load), which is possibly related to its immunosuppressive effect. The duration of viraemia also appeared to be prolonged (median time to undetectable, 12 vs 8 days), though the difference did not reach statistical significance. Our study was limited by a small sample size. Patients with advanced age, co-morbidity, and those immunocompromised were excluded. Moreover, viral load profiles among more severe SARS cases, and the clinical consequence of a higher plasma viral load in early hydrocortisone treated patients needs further investigation.

To increase the throughput of a previously developed quantitative serum SARS-CoV RNA RT-PCR assay,[4,5] we evaluated the feasibility of automating the RNA extraction procedure through the use of the MagNA Pure LC instrument (Roche Diagnostics). Reagent kits suitable for the extraction of viral RNA from serum and plasma as recommended by the instrument manufacturer were evaluated. As the extraction procedure needed to conform to the biosafety practices recommended by the World Health Organization (WHO), a modified protocol incorporating an external lysis-processing step for the MagNA Pure LC total nucleic acid large volume kit (Roche Diagnostics) was developed. The WHO recommends that nucleic acid extraction procedures for SARS-CoV involving untreated specimens should first be performed under biosafety level-2 facilities, with additional level-3 work practices. After the viral particles had been lysed or inactivated, the specimens could be handled according to standard level-2 practices. We showed that the use of the large volume kit resulted in better analytical sensitivity when compared with the total nucleic acid kit, as evident by higher rates of positive detection among samples containing low concentrations of SARS-CoV. Furthermore, the MagNA Pure LC system was shown to be free from problems of carry-over contamination.

The automated extraction method involving the use of the large volume kit with the external lysis procedure was further compared with the quantitative performance of a previously described manual viral RNA extraction method based on the use of the QIAamp viral RNA mini kit (Qiagen). Across a wide range of SARS-CoV concentrations in both transport medium and serum, viral RNA extracted from the automated method led to SARS-CoV concentrations that were consistently lower than when extracted by the manual method. Furthermore, better detection rates were observed for serum containing low concentrations of SARS-CoV extracted manually than by the automated method. The manual method also contributed to better overall analytical precision as evident by the lower coefficients of variation.

Our study clearly demonstrated the feasibility of using allelic discrimination assays as a method for genetic characterisation of SARS-CoV genotypes in patients. It was particularly useful when extensive sequence information was available. Direct sequencing is still the gold standard for identifying new sequence variations when new infectious disease agents continue to emerge and old ones re-emerge. Once the variations have been identified, allelic discrimination assay is more efficient and suitable for large-scale population investigations. Thus, this approach provides a rapid and simple means to perform accurate genotype screening, making it ideal for epidemiological investigations.

Conclusions

Our study demonstrated that 'early' corticosteroid treatment was associated with a higher subsequent plasma viral load and therefore should be avoided. The automated viral RNA extraction protocol was less sensitive, less precise and produced quantitative results that were consistently lower than those of column-based manual extraction. We have evaluated a rapid approach for characterising SARS-CoV genotypes. The assay is simple, easy to perform and reproducible.

Judicious use of corticosteroid therapy in SARS is advisable. As it has been previously shown that the serum SARS-CoV concentration has prognostic implications and serial assessment is useful for monitoring patient progress, the superior quantitative performance and precision of the column-based extraction are additional reasons for favouring its use rather than the automated protocol. The rapid genotyping method based on TaqMan allelic discrimination can therefore be used as an efficient means to screen for virus genotypes and track the transmission of a particular viral strain during epidemics.

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1. Lee N, Allen Chan KC, Hui DS, et al. Effects of early corticosteroid treatment on plasma SARS-associated coronavirus RNA concentrations in adult patients. J Clin Virol 2004;31:304-9.
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Proteomic profiling in SARS: diagnostic and prognostic applications

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Key Messages

1. Disease-specific proteomic fingerprints were found in SARS patients.
2. The two proteomic features yielding the largest receiver operating characteristic curve area (diagnostic accuracy of >95%) were an N-terminal fragment of complement C3c α-chain (m/z 28119) and an internal fragment of fibrinogen alpha-E chain (m/z 5908).
3. In contrast to previous proteomic studies, we found that serum amyloid A was not useful in the diagnosis of SARS.
4. The potential prognostic features of m/z 7768 and m/z 8865 were found to be platelet factor 4 and beta-thromboglobulin, respectively.

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Introduction

Advances in proteomics have provided new strategies to identify biomarkers and therapeutic targets, and to study the pathology of diseases. Surface-enhanced laser desorption/ionisation (SELDI) ProteinChip technology is a proteomic tool that has been applied to the discovery of diagnostic proteomic fingerprints for various diseases, including cancer and infectious diseases.[1-3] This technology has been used to identify potential biomarkers for early diagnosis of SARS.[1,4-6] In these studies, the controls were either healthy subjects or persons with non-SARS viral infection. Regrettably, the similarity of the symptoms between SARS and control patients, and the time point of blood collection were not considered. From the perspective of infectious disease diagnosis, one should identify the disease causing the symptoms in patients presenting with similar symptoms, not differentiate healthy subjects from infected patients.[7]

We compared the serum proteomes between SARS and non-SARS patients, and identified the potential protein marker for diagnosis and prognosis of SARS. The non-SARS patients were those who had similar symptoms to SARS patients. They were admitted to the same hospital and were later shown to be negative for SARS-CoV infection. For both SARS and non-SARS patients, sera were collected within 1 week of the fever onset.

Aims and objectives

1. To characterise the proteomic fingerprints of SARS or specific proteomic features in serum of SARS patients;
2. To investigate if the serum proteomic profiles are useful in early diagnosis of SARS;
3. To investigate if the variations of the serum proteomic profile correlate with clinical events;
4. To investigate if the serum proteomic profiles are of prognostic significance in SARS patients; and
5. To uncover the protein identity of serum proteomic features with potential diagnostic and prognostic value.

Methods

Patients

The SARS group included 13 males and 26 females; the mean age was 42 (range, 21-88) years. The non-SARS group included 18 males and 21 females; the mean age was 44 (range, 20-88) years. The pre-treatment serum samples from both groups represented the first time point after hospitalisation (3-7 days from onset of fever). All the SARS cases were positive for anti-SARS-CoV IgG antibody. The non-SARS patients were controls who had similar symptoms as the SARS patients and were admitted to the same hospital and later shown to be serologically negative for anti-SARS-CoV antibody even 6 weeks after the onset of symptoms.

Serum proteomic profiling

For all the SELDI ProteinChip analyses, the serum samples from the diseased and control groups were randomised and the investigator was blinded. The SELDI ProteinChip analysis was performed as previously described,[2,4,7] using CM10

ProteinChip arrays (Ciphergen Biosystems). Two binding conditions were performed: at pH 4.0 and pH 9.0.

Bioinformatic analysis

The significance analysis of microarray (SAM) algorithm (Stanford University, CA, US) was used to identify proteomic features with levels significantly different between the SARS and non-SARS patients.[2,4,7] Correlations between the differential proteomic features and various clinical and biochemical features were examined by the Spearman rank-order correlation test. Significantly differential proteomic features correlated with various clinical/biochemical correlations were then subjected to two-way hierarchical clustering analysis, as previously described.[2]

Protein purification

For protein identification, proteins corresponding to the SEDLI peaks were purified by cation exchange chromatography with the use of CM10 ceramic beads (BioSepra) under the binding conditions similar to those for CM10 ProteinChip arrays. The purified proteins were resolved by two-dimensional gel electrophoresis. Protein spot with mass matched with the differential proteomic feature was excised and subjected to mass spectrometry (MS) analysis.

Protein identification

Protein spots of interests were removed from the gel and subjected to trypsin digestion as previously described.[8] The trypsin digests were then extracted and subjected to tandem MS analysis using the ABI 4700 system (Applied Biosystems). The fragment masses and intensities of each MS/MS mass spectrum were subjected to online Mascot MS/MS ion search (http://www.matrixscience.com/) to determine the protein identities.

Results

Identification of differential serum proteomic features

The serum proteomic profiles of 39 SARS and control patients were obtained, and 820 common proteomic features were found. At a median false discovery rate of zero (SAM analysis), levels of 107 serum proteomic features were significantly different between the SARS and control patients. In SARS patients, 52 and 55 proteomic features were present at higher and lower levels, respectively. Among these 107 differential proteomic features, 20 yielded significant correlations with two or more clinical/biochemical parameters. As a result, there were 20 potential biomarkers for the detection of SARS; in SARS patients 15 and 5 yielded positive and negative correlations, respectively. Hierarchical clustering analysis showed that these 20 biomarkers contained information to identify SARS patients at high accuracy (sensitivity=95%, specificity=100%), SARS patients with a poor prognosis (ie requiring care in the intensive care unit or supplementary oxygen).

Correlation with clinical/biochemical parameters

The biomarker of m/z 24504 correlated positively with SARS coronavirus load, whereas that of m/z 4680 correlated negatively with viral load. Ten biomarkers correlated positively with C-reactive protein, suggesting their levels were affected by the acute phase reaction response. Whereas 12 others correlated positively with lactate dehydrogenase levels, which suggested they were associated with the lung damage. Two biomarkers correlated positively with serum albumin and/or total protein levels, indicating an association with the liver function. Whereas 13 others correlated negatively with albumin and/or total protein (but not alanine transaminase), reflecting the effect of decrease in liver function, but their presence may not have been due to liver damage. Three biomarkers correlated positively with age. Ten biomarkers correlated positively (one negatively) with neutrophil counts.

Diagnostic values of the proteomic biomarkers

Receiver operating characteristic (ROC) curve analyses showed that all the differential proteomic features were potential biomarkers for identifying SARS patients. The ROC curve areas of all the 20 biomarkers were in the range of 0.733 to 0.955. For example, the ROC curve for the peak intensity of biomarker m/z 28120 was 0.987 (95% confidence interval [CI], 0.966-1.007). At a specificity of 97%, its sensitivity was 97%. The ROC curve for 1/peak intensity of biomarker m/z 5908 was 0.995 (95% CI, 0.985-1.004). At a specificity of 95%, its sensitivity was 100%.

Analysis of the diagnostic value of serum amyloid A

This SELDI proteomic feature corresponding to serum amyloid A was not identified to be a potential diagnostic marker. This finding was confirmed by immunoassay.

Prognostic values of the proteomic biomarkers

By multivariate logistic regression, we analysed the prognostic values of the 20 SARS-associated proteomic features and 10 serological variables (alanine transaminase, lactate dehydrogenase, bilirubin, total protein, albumin, globulin, C-reactive peptide, total white blood cell count, lymphocyte count, and neutrophil count) in pretreatment samples from 38 SARS patients. Serum proteomic features of m/z 6634 (P=0.010), m/z 7768 (P=0.017) and m/z 8865 (P=0.045) were significantly associated with supplemental oxygen usage by the patients, whereas a proteomic feature of m/z 8635 (P=0.016) was associated with admission to intensive care units.

Purification and identification of the proteomic biomarkers with diagnostic/prognostic values

The proteins corresponding to the differential proteomic features were purified and separated by chromatographic and gel electrophoresis techniques. The purified proteins were subjected to mass spectrometric analysis to identify the proteins. Protein identities of six diagnostic and prognostic proteomic features were obtained (Table).

Table. Protein identities of six diagnostic and prognostic proteomic features using surface-enhanced laser desorption/ionisation (SELDI) ProteinChip technology

SELDI peak (m/z)	Protein identity	SwissProt entry no.	Higher/lower levels in SARS than control patients
5908	Internal fragment of fibrinogen alpha-E chain	P02671	Lower
7768	Platelet factor 4	P02776	Lower
8865	Beta-thromboglobin	P02775	Higher
24500	Ig Kappa light chain	223335 (NCBI)	Higher
28120	N-terminal fragment of complement C3c	P01024	Higher
88650	Immunoglobin heavy constant gamma 1	P01857	Higher

Discussion

Two studies reported potential biomarkers in the sera of adult SARS patients using the SELDI ProteinChip technology.[1,5] In the present study, the intensity of the proteomic feature of m/z 7769 was significantly lower in SARS patients (Mann Whitney test, $P<0.001$), as noted in another study (Mann Whitney test, $P=4.9\times10^{-8}$).[1] Other SARS-associated proteomic features differed, probably due to different selection criteria for the control subjects. In previous studies, the controls were either healthy subjects or patients from other clinics with viral infections. The degree of similarity of the symptoms between SARS and control groups, and the time point of blood collection were not considered. In the present study, the controls were suspected SARS patients admitted to the same hospital as SARS patients, but later shown to be negative for SARS-CoV infection. The symptoms and the time points for blood sampling were similar in SARS and control patients. Thus, the biomarkers identified in the present study may be more advantageous in actual diagnostic settings than those identified in previous studies.

The different findings reported in various studies could also be due to the use of different profiling methodologies. In a previous study, a comprehensive profiling approach was used.[1] After denaturing with urea and detergent, the serum proteins were first fractionated with anion exchange beads to give six fractions, which were later analysed with arrays involving copper ProteinChips and weak cation exchange CM10 ProteinChips. The comprehensive profiling approach increases the chance of identifying more potential protein markers.[2] In the present study, we analysed the serum proteins directly, using only the CM10 ProteinChip arrays at two different binding conditions (pH 4 and pH 9). We chose the CM10 ProteinChip arrays (previously called WCX2) because its chip type was shown to give the best profiling when analysing serum samples from the SARS patients.[5] Although the direct binding approach might lead to the discovery of fewer biomarkers, such assays have a higher potential for modification for a clinical assay even without knowing the protein identities of the disease-specific SELDI peaks.

In previous studies, platelet factor 4 (PF4) and beta-thromboglobin (beta-TG) were found to be chemokines involved in the pathogenesis of acute respiratory distress syndrome (ARDS) in a negative and positive manner, respectively. The computed tomographic features of ARDS caused by SARS are similar to those ARDS caused by other causes. In SARS patients, low serum levels of PF4 and high serum levels of beta-TG were associated with a poor prognosis. PF4 and beta-TG may be important chemokines involving the development of ARDS in SARS patients.

Conclusions

Specific proteomic fingerprints were present in the sera of adult SARS patients. They could be used to identify SARS cases during early onset of the disease with high specificity and sensitivity, and could also be used for prognosis. The proteins with potential diagnostic and prognostic values were successfully identified. The SELDI ProteinChip assay could be used for first-line detection of SARS, followed by a quantitative viral RNA assay for confirmation. Once confirmed, the treatment strategy could be adjusted according to the anticipated prognosis, based on the SELDI ProteinChip profiling and the viral RNA level. As the protein identities of the proteomic features with diagnostic and prognostic values have been identified, in the future specific immunoassays may be developed for the diagnosis of SARS and to offer a prognosis.

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1. Pang RT, Poon TC, Chan KC, et al. Serum proteomic fingerprints of adult patients with severe acute respiratory syndrome. Clin Chem 2006;52:421-9.
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Functional roles of 3a protein in the pathogenesis of SARS

Key Message

The SARS-CoV 3a protein can induce apoptosis through a caspase-8-dependent pathway in VeroE6 cells.

Introduction

Severe acute respiratory syndrome (SARS) affected more than 8000 individuals and resulted in about 800 deaths in 26 countries. The genomes of different strains of SARS-CoV have been sequenced and found to contain 15 open reading frames (ORFs) encoding the replicase, four major structural proteins and several proteins of unknown function.[1-3]

The *3a* locus encodes one of the ORFs of unknown function and is located between two structural genes encoding the spike and the envelope proteins of SARS-CoV. Interestingly, the *3a* ORF is not found in any coronaviruses identified to date. This suggests that the 3a protein is a newly emerged protein in coronaviruses.

Previous studies have shown that many coronaviruses, including murine hepatitis virus, avian infectious bronchitis virus and transmissible gastroenteritis coronavirus, are able to induce apoptosis of host cells,[4] but little is known about this ability in SARS-CoV. Apoptosis was observed in liver specimens from patients with SARS-associated viral hepatitis, just as lymphopenia is commonly observed in SARS patients (postulated to be due to apoptosis induced by SARS-CoV infection).[5,6] Furthermore, SARS-CoV can induce a cytopathic effect and apoptosis in cell-culture models, such as VeroE6 cells.[7]

Aims and objectives

1. To identify the molecular mechanism underlying the 3a-induced apoptosis in SARS-CoV infected VeroE6 and human cells.
2. To identify potential inhibitors of such 3a-induced apoptosis.

Methods

The cDNA coding for the SARS-CoV 3a protein was cloned into mammalian expression vectors pcDNA4 and pEGFP and expressed in VeroE6 cells. Apoptosis induced by the 3a protein expression was detected by a DNA fragmentation assay, chromatin-condensation analysis and immunostaining and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assay. To dissect the signalling pathway mediating the 3a-induced apoptosis, the expression of apoptosis-related proteins were determined by western blot analysis. We have also employed the commercially available antibody array to identify more protein targets involved in 3a-induced apoptosis.

Results

To investigate whether the 3a protein could induce apoptosis, Vero E6 cells were transfected with pEGFP-3a. Extensive chromatin condensation was observed in green fluorescent protein-positive cells. To examine whether the 3a protein induced DNA fragmentation, Vero E6 cells were transfected transiently with pcDNA4-3a. Extensive low-molecular-mass apoptotic DNA fragments were observed on day 3 onwards after transfection. The apoptotic effect of the 3a protein was finally confirmed by the TUNEL assay.

To delineate the pathway by which the 3a protein might be involved in the

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induction of apoptosis, we examined the expression levels of Bcl-2 family proteins and caspase-8, which are common mediators of the mitochondrion- and receptor-mediated pathways, respectively. Cleavage of procaspase-8 was increased in 3a-transfected Vero E6 cells. However, there were no effects on the endogenous levels of Bcl-2 family proteins (such as Bcl-2 and Bad) and on proliferating-cell nuclear antigen.

Using the commercially available antibody array, many apoptosis-related genes, including β-catenin, cytochrome c, caspase 4, glycogen synthase kinase 3 beta, Fas-associated death domain protein, p53 binding protein 2, and protein kinase R were upregulated in VeroE6 cells expressing the SARS-CoV 3a protein. These may be novel target genes triggered by the SARS-CoV 3a protein.

Discussion

Our study has shown that expression of the 3a protein can induce chromatin condensation and low-molecular-mass apoptotic DNA fragmentation from 3 days post-transfection. These data were consistent with the results of the TUNEL assay, showing a significant amount of internucleosomal DNA cleavage. Since caspase-8 was activated in 3a-induced apoptosis, we postulate that expression of the 3a protein induces apoptosis through a pathway similar to the death-receptor signalling cascades. Finally, additional proteins targets were identified by antibody array for future investigation of SARS-CoV induced apoptosis.

Conclusions

The 3a protein can induce apoptosis thorough a caspase-8-dependent pathway in VeroE6 cells. An anti-apoptotic strategy can be considered in future outbreaks of SARS or SARS-related diseases.

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Long-term sequelae of SARS: physical, neuropsychiatric, and quality-of-life assessment

Key Messages

1. Impairment of lung diffusing capacity persisted in 24% of SARS survivors; their exercise capacity and health status were markedly lower than the general population at 1 year after illness onset.
2. There was no difference in lung function indices, exercise capacity, and health status at 1 year between the intubated and non-intubated SARS patients admitted to the intensive care unit, although the former had more severe lung injury.
3. The functional disability in SARS survivors appears out of proportion to the degree of lung function impairment and may be due to additional factors such as muscle deconditioning, steroid-related musculoskeletal complications, critical illness-related neuropathy/myopathy, and/or psychological factors.

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Introduction

The emergence of SARS in Southern China in November 2002, followed by the global outbreak in 2003, caught the medical profession by surprise.[1] Studies on SARS-coronavirus viral loads have shown that peak viral levels were reached at the second week of illness when patients were in hospital care, and thus health care workers (HCWs) were particularly prone to infection.[2,3] About 20 to 36% of SARS patients were admitted to the intensive care unit (ICU), whereas 13 to 26% progressed to acute respiratory distress syndrome (ARDS) and received invasive ventilatory support.[2,4,5]

In our hospital, over half of those infected were HCWs.[4] At 5 weeks after discharge, high-resolution computed tomography for 24 out-patients with residual radiological opacities revealed multiple patchy ground glass appearance and interstitial thickening (n=9, 38%) and fibrotic changes (n=15, 62%).[6] Serial lung function, exercise capacity, chest radiographs, and health-related quality of life (HRQoL) were examined at 3, 6, and 12 months after illness onset, and SARS survivors who had been admitted to the ICU were compared to those who were only treated in medical wards.[7,8]

Aims and objectives

To examine the impact of SARS on pulmonary function, exercise capacity, and HRQoL among survivors.

Methods

This was a prospective longitudinal follow-up study of patients with SARS discharged from our hospital after surviving the outbreak in 2003. The patients came from our previously reported cohort[4] recruited over a period of 2 weeks from 11 to 25 March 2003. All patients in this study had laboratory-confirmed SARS.[9]

Following discharge, lung functions of patients were evaluated at the end of 3, 6 and 12 months after disease onset. Subjects were interviewed and underwent physical examination, pulmonary function testing, respiratory muscle strength measurement, posteroanterior chest radiography, resting oximetry, and a standardised 6-minute-walk (6MW) test. In addition, they completed the Medical Outcomes Study 36-item Short-Form General Health Survey (SF 36) to measure HRQoL. The 6MW distances obtained for each patient on 2 separate days were compared to the normative reference data collected from a population survey of 538 normal healthy subjects in 2004 by the Coordinating Committee in Physiotherapy of the Hong Kong Hospital Authority.[7,8]

Lung volumes (total lung capacity [TLC], vital capacity [VC], residual volume [RV], functional residual capacity [FRC] using the nitrogen washout method), spirometry (forced vital capacity [FVC], forced expiratory volume in one second [FEV_1], FEV_1/FVC ratio, forced expiratory flow rate over middle 50% of FVC [FEF_{25-75}]), and surface area for gas exchange (diffusion capacity adjusted for haemoglobin [DLCO] and DLCO per alveolar volume [K_{CO}]) were

performed with the Vmax System (SensorMedics Corp, CA, USA). The DLCO was determined by the single-breath carbon monoxide technique using an infrared analyser. The results were compared to available normative data[10] widely adopted as reference values in Hong Kong before 2006.

Results

Of the first 138 patients infected with SARS in March 2003, 15 (11%) died.[4,9] Among the 123 survivors, 13 (11%) did not attend for follow-up at 3 and 6 months,[7] whereas another 13 (11%) defaulted the 12-month assessment.[8] Thus, 44 males and 66 females with a mean age of 36 (standard deviation [SD], 10) years and body mass index of 23 (SD, 5) kg/m^2 completed the 6-month assessment. Seventy (64%) of them were HCWs.

At 6 months, 33 (30%) of the subjects had abnormal chest radiographs. Four (4%), 8 (7%), and 17 (16%) patients had FVC, TLC, and DLCO values below 80% of predicted, respectively; whereas 15 (14%) and 24 (22%) had Pimax and Pemax values below 80 cm H_2O, respectively. The mean 6MW distance increased from 464 (SD, 83) m at 3 months to 502 (SD, 95) m (95% confidence interval [CI], 22-54 m, P<0.001), but this distance was shorter than in normal controls in the same age-groups, indicating impairment of HRQoL at 6 months.[7]

At 1 year, 39 males and 58 females completed the serial assessments; 63 (70%) of them were HCWs with a mean age of 40 (SD, 10) years and mean body mass index of 24 (SD, 4) kg/m^2. Twenty-seven (28%) of the patients had abnormal chest radiographs. Four (4%), 5 (5%), and 23 (24%) of the patients had FVC, TLC, and DLCO values below 80% of predicted, respectively. The mean 6MW distance was 511 (SD, 90) m, which was higher than that at 3 months (mean difference, 47; 95% CI, 32-62 m; P<0.01) but not different from that at 6 months (mean difference, 10; 95% CI, -4 to 24 m, P=0.18). The 6MW distance of the survivors was less than that in normal controls of the same age-groups, indicating impairment of HRQoL at 12 months. Patients admitted to the ICU (n=31) had higher mean chest radiograph scores (1.6 [SD, 3.1] vs 0.4 [SD, 1.1], P=0.04) and lower % predicted FVC, TLC, and DLCO values than those not admitted to the ICU, but there were no significant differences with respect to their 6MW distance and health status.[8]

Discussion

This prospective cohort study has shown that 24% and 28% of SARS survivors had impaired lung diffusing capacity and abnormal chest radiographs, respectively, at 1 year after illness onset. Overall, the serial assessments of 6MW distance revealed significant improvement over 12 months, though exercise capacity and health status were still significantly lower than in normal controls of the same age-groups. The 1-year lung function indices in SARS survivors who were admitted to ICU were markedly inferior to those who were only treated on medical wards, although no significant differences were noted for the 6MW distances, respiratory muscle strength, and health status between the two groups. Interestingly, there was no difference in lung function indices, exercise capacity, and health status at 1 year between intubated and non-intubated ICU SARS patients, although the former had more severe lung injury.[8]

Despite the presence of extensive parenchymal changes on computed tomography during the early convalescent period,[6] surprisingly most lung function test indices of SARS patients were within normal limits in most of our patients.[7,8] Their poor exercise performance appeared out of proportion to the degree of lung function impairment and may be due to extra-pulmonary factors such as muscle deconditioning, steroid or viral-induced myopathy, critical illness polyneuropathy/myopathy,[11] and/or other psychological factors. Eighteen out of 44 SARS survivors in Singapore had reduced exercise capacity at 3 months after discharge, which could not be accounted for by impairment of pulmonary function.[12] The inability to exercise in their recovered SARS patients was primarily due to extra-pulmonary causes such as physical deconditioning and steroid myopathy. Among our SARS survivors, at 3 months after illness onset, strength and endurance were more impaired in proximal than distal muscles.[13]

Among SARS survivors, persistent lung function abnormalities occurred in less than one third of patients at 1 year, and yet there was a significant impairment of health status.[8,14-16] The results are not surprising, as these patients endured long periods of isolation and extreme uncertainty during the SARS illness that could have created enormous psychological stress[17] and mood disturbances,[18] in addition to the physical impairment. In addition, steroid toxicity, personal vulnerability, and psychosocial stressors might have jointly contributed to the development of psychosis in some patients.[19] Longer term follow-up is needed to assess whether these effects persist.

Conclusions

Significant impairment of diffusing capacity persisted in 24% of SARS survivors; at 12 months after illness onset their exercise capacity and health status were markedly diminished compared to the general population. Further follow-up is needed to assess if these deficits persist.

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1. Hui DS, Joynt GM, Wong KT, et al. Impact of severe acute respiratory syndrome (SARS) on pulmonary function, functional capacity and quality of life in a

cohort of survivors. Thorax 2005;60:401-9.

2. Hui DS, Wong KT, Ko FW, et al. The 1-year impact of severe acute respiratory syndrome on pulmonary function, exercise capacity, and quality of life in a cohort of survivors. Chest 2005;128:2247-61.

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Correlation of clinical outcomes and radiographic features in SARS patients

Key Message

In SARS patients, more extensive airspace disease on chest radiographs at presentation is an independent predictor of adverse outcome (admission to the intensive care unit or death).

Introduction

In March 2003, there was a major outbreak of SARS in Hong Kong. In the Prince of Wales Hospital, 138 patients including health care workers contracted the disease. Of these patients, 23% progressed to acute respiratory failure for which they were admitted to the intensive care unit (ICU), whereas 14% of the 138 patients received invasive mechanical support within 1 to 2 weeks.[1] Chest radiography remains the first-line radiological investigation in suspected cases and helps monitor progress during treatment. Air-space opacity in peripheral and lower-zone distribution was most commonly seen at presentation. It then progressed to multi-focal or bilateral lung involvement; about 70% of SARS patients showed improvement.[2]

Severity of lung abnormalities on chest radiographs correlates positively with clinical and laboratory parameters, such as SaO_2 and liver enzymes, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels.[3] Moreover, there are correlations between radiographic parameters, oxygen supplementation, and apparent response to treatment.[4] We aimed to evaluate the correlation between clinical outcome and the radiographic features of SARS patients.

Methods

Patients

A cohort admitted from 11 to 25 March 2003 were analysed.[1] There were 66 males and 72 females with a mean age of 39 (standard deviation [SD], 17) years. All patients were ethnic Chinese. Clinical and laboratory data were recorded up to 5 April 2003.

Diagnosis and monitoring of progress

The diagnosis of SARS was based on the Centers for Disease Control criteria.[5] Initial investigations included complete blood count, clotting profile (prothrombin time [PT], activated partial thromboplastin time [APTT], international normalised ratio [INR], D-dimer) and serum biochemistry (including electrolytes, renal function test, liver function test, creatinine kinase [CPK], lactate dehydrogenase [LDH]). The INR was measured because some patients might develop disseminated intravascular coagulation. These parameters and chest radiographs were monitored daily. Resort to supplemental oxygen and timing of pulse methylprednisolone during the study period were also recorded.

Treatment

Patients who developed hypoxia were given oxygen therapy through nasal cannulae. Patients were admitted to the ICU when they developed respiratory failure as evidenced by: (1) failure to maintain an arterial oxygen saturation of at least 90% while receiving supplemental oxygen of 50%, and/or (2) a respiratory rate greater than 35 breaths per minute. Nineteen (14%) patients received invasive mechanical ventilation.

Radiographic assessment

Anteroposterior chest radiographs of the 138 patients were taken at presentation

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and daily during the hospital stay. All 2045 radiographs were assessed (a mean of 15 per patient; range, 3-26) by three radiologists (who were unaware of the clinical progress) and consensus reached. Each lung was divided into three zones (upper, middle and lower). Each zone spanned one third of the craniocaudal distance of the lung and was evaluated separately. The presence, appearance, distribution and size of lung parenchymal abnormalities on each radiograph were recorded. The area (%) of lung involved in each zone was estimated visually; the maximum % of each zone was 100%. The overall mean % of lung parenchymal involvement of the six lung zones could range from 0% to 100%.[2]

The progression pattern based on serial chest radiographs was categorised into four patterns according to our previous study.[2] Type 1 referred to: initial deterioration to peak level followed by improvement, peak level defined as overall mean lung involvement of >25% of the initial extent. Type 2 referred to: fluctuating radiographic changes, with at least two peaks and an intervening trough, trough level defined as overall mean lung involvement differing from the peak level by >25%. Type 3 referred to: static radiographic changes, with no apparent peak (ie change in overall mean lung involvement <25% of the initial extent) for more than 10 days. Type 4 referred to: progressive radiographic deterioration with no improvement.

Table 1. The extent of consolidation on initial chest radiographs of 138 SARS patients

Extent of consolidation (%)	No. (%) of patients
0	30 (22)
0.1-2.5	58 (42)
2.6-5.0	29 (21)
5.1-10	10 (7)
10.1-15	6 (4)
>15	5 (4)

Table 2. The number of lung zones involved on initial chest radiographs of 138 SARS patients

No. of lung zones involved*	No. of patients	Median (interquartile range) interval of chest radiographs taken after fever onset (days)
0	30	1.5 (0-5)
1	59	2.0 (0-9)
2	28	2.0 (0-10)
3	12	3.5 (0-10)
4	6	2.5 (0-6)
5	1	3.0 (3-3)
6	2	3.5 (0-7)

* Each lung is divided into three zones: upper, middle, and lower. Each zone spans one third of the craniocaudal distance of the lung on an anteroposterior chest radiograph

Results

Findings on chest radiograph

On average the initial chest radiograph was taken 2.5 days after the onset of fever (range, 0-10 days). A total of 108 of the 138 (78%) patients had air-space opacity at presentation; 59 (55%) had focal unilateral opacities and 49 (45%) had unilateral multi-focal or bilateral involvement. Initial radiographs of 30 (22%) patients were normal, but 29 of them had air-space opacities on follow-up radiographs 1 to 7 days (median, 3 days) later.[2] At presentation, the overall mean lung involvement was 5% (range, 1-63%).[2] The extent of consolidation and number of zones involved on the initial radiographs are summarised in Tables 1 and 2, respectively.[6] Regarding the pattern of progression, type 1 was the most common (97/138, 70%), followed by type 2 (24/138, 17%), type 3 (10/138, 7%), and type 4 (7/138, 5%).[2]

The peak of the extent of pneumonic changes corresponded to the time of commencement of pulse intravenous methylprednisolone treatment. Consolidation peaked at a mean interval of 9 (SD, 3) days after fever onset. The median time of starting the first pulse of methylprednisolone was 8 days after fever onset (interquartile range [IQR], 6-9 days).

Laboratory results

The initial blood counts showed leukopaenia (total white cell count of <3.5 x10^9/L; normal range [NR], 3.5-10.5 x10^9/L) in 47 (34%) of the patients. The neutrophil count was within normal limits in most cases (median, 3.5 x10^9/L; range, 0.5-11.8 x10^9/L; NR, 1.5-6.6 x10^9/L), but a moderate lymphopaenia (absolute lymphocyte of <1.0 x10^9/L; NR, 1.0-3.5 x10^9/L) was present in 96 (70%) of the patients.[1] Biochemistry measurements revealed elevated serum ALT in 32 (23%) of the patients (mean, 60.4; SD, 150.4; NR, <55 IU/mL); CPK was elevated in 44 (32%) of the patients (median, 126; range, 29-4644; NR, 42-218 U/L), whereas the LDH was elevated in 98 (71%) of the patients. At presentation, the mean LDH was 287.7 (SD, 143.3; NR, 87-213) U/L for those not admitted to the ICU and 558 (SD, 258) U/L for those admitted to the ICU or dead (P<0.001). The mean peak LDH was 310 (SD, 153.8) U/L for the former and 629.7 (SD, 283.5) U/L for the latter (P<0.001).[1]

Clinical outcomes

Of the 138 patients, 36 (26%) were admitted to the ICU due to respiratory failure. In the first 4 weeks of the outbreak, there were eight deaths (crude mortality rate=6%) related to severe respiratory failure: six died in the ICU and two on medical wards; all were originally admitted for other major medical conditions. In total, 38 patients reached the clinical end-point for poor outcome (ie ICU admission or death).

Correlation between clinical outcome and radiographic features

Compared to survivors not admitted to ICU, those admitted

Table 3. Correlation between clinical outcomes and radiographic features*

Radiographic feature	Patients who received ICU care and/or died	Surviving patients who did not receive ICU care
Median (interquartile range) extent of consolidation (%)		
Day 0	3.3 (1.7-8.8)	1.7 (0.0-3.3)
Day 7	15.0 (6.5-28.7)	5.0 (2.5-7.5)
No. of lung zones involved		
Day 0		
≤1 (n=89)	14 (16)	75 (84)
>1 (n=49)	24 (49)	25 (51)
Day 1		
≤1 (n=56)	3 (5)	53 (95)
>1 (n=82)	35 (43)	47 (57)
Consolidation at day 0		
Unilateral (n=67)	13 (19)	54 (81)
Bilateral (n=41)	22 (54)	19 (46)
Progression pattern		
Type 1 (n=97)	17 (18)	80 (83)
Types 2-4 (n=41)	21 (51)	20 (49)

* Unless otherwise stated, data are presented as no. (%) of patients

to ICU or died had more extensive radiographic evidence of pneumonia on the initial-day (median, 1.7% vs 3.3%; IQR, 0-3.3% vs 1.7-8.8%; P<0.001) and day-7 (after fever onset) radiographs (median, 5% vs 15%; IQR, 2.5-7.5% vs 6.5-28.7%; P<0.001, Table 3).

Compared to those with one or less than one zone involved, those with more than one zone involved on the initial day (14/89 vs 24/49, P<0.001) and day 7 (3/56 vs 35/82, P<0.001) were significantly more likely to have been admitted to the ICU or dead (Table 3).

Patients with bilateral pneumonic changes on the initial radiograph were more likely to have been admitted to the ICU or dead than those with unilateral involvement (22/41 vs 13/67, P<0.001, Table 3).

Among the 97 patients with type-1 radiographic pattern, only 17 (18%) were admitted to the ICU or dead, as opposed to 21 (51%) of the 41 patients with types 2 to 4 patterns (P<0.001). Of these 21 patients, 14 had type-2 and seven type-4 patterns (Table 3).

The cumulative % of patients with SARS not receiving supplementary oxygen versus the extent of consolidation is shown in the Kaplan-Meier curve in the Figure.[6]

The rate of change of LDH (units/day) correlated with the rate of change of % involvement in chest radiographs (Spearman r_s=0.40, P=0.014).

Univariate analysis revealed that advanced age, male gender, peak CPK value, LDH at presentation and its peak value, higher initial absolute neutrophil counts, and low serum sodium levels were predictive factors for ICU admission and death.[1] Following multivariable analysis, other independent predictors of adverse outcomes were identified.[6] These included: the number of zones involved in initial chest radiographs, advanced age (odds ratio [OR] per year=1.1; 95% CI=1.0-1.1; P<0.001), high baseline LDH (OR per U/L=1.0; 95% CI=1.0-1.0; P=0.001), higher absolute neutrophil counts at presentation (OR=1.4; 95% CI=1.0-1.8; P=0.025), and more than one zone involved in initial chest radiographs (OR=3.2; 95% CI=1.1-9.3; P=0.037).

Discussion

SARS was a new infectious disease with a high morbidity and mortality. It usually progressed to acute respiratory failure within a week, with radiographic evidence of air-space disease. Chest radiography (supplemented by thin-section computed tomography of the thorax in selected cases) is a useful diagnostic and management tool. At presentation, 78% of our patients had evidence of air space consolidation on their chest radiographs. Initial radiographs were normal in over 20% of patients, but follow-up radiographs showed abnormality at a median of 3 days.[2]

In some patients, consolidation may progress to respiratory failure, ICU admission, and/or death. Progression of consolidation peaked at a mean of 9 (SD, 3) days from fever onset. There appeared to be a strong correlation between the extent of radiographic abnormality and the degree of respiratory failure. This can be inferred from the Kaplan-Meier plot (Fig). Even if only a small percentage (10%) of the lung showed consolidation, approximately 50% of such patients received supplementary oxygen. Based on evidence of progression on serial chest radiographs, intravenous pulse methylprednisolone was administered in order to control immune-mediated lung injury; the median time of commencement was 8 days from the onset of symptoms. Although a favourable clinical response appeared to have been achieved in most patients, 36 (26%) received ICU care and eight died.

In view of the high proportion of SARS patients receiving ICU care, it would have been desirable to have parameters that help predict clinical outcome. In that respect, the radiographic extent of pneumonia at presentation appeared to correlate with adverse clinical outcomes. More extensive air-space diseases (as reflected by a higher % of consolidation, more than one zone being involved in chest radiographs on admission and on day 7 after fever onset, and bilateral disease) were associated with subsequent ICU admission or death. This finding is similar to other forms of community-acquired pneumonia in general, including the ICU-treated populations with abnormal air-space opacities in both lungs, more than one lobe involved, and rapid radiographic progression, all of which

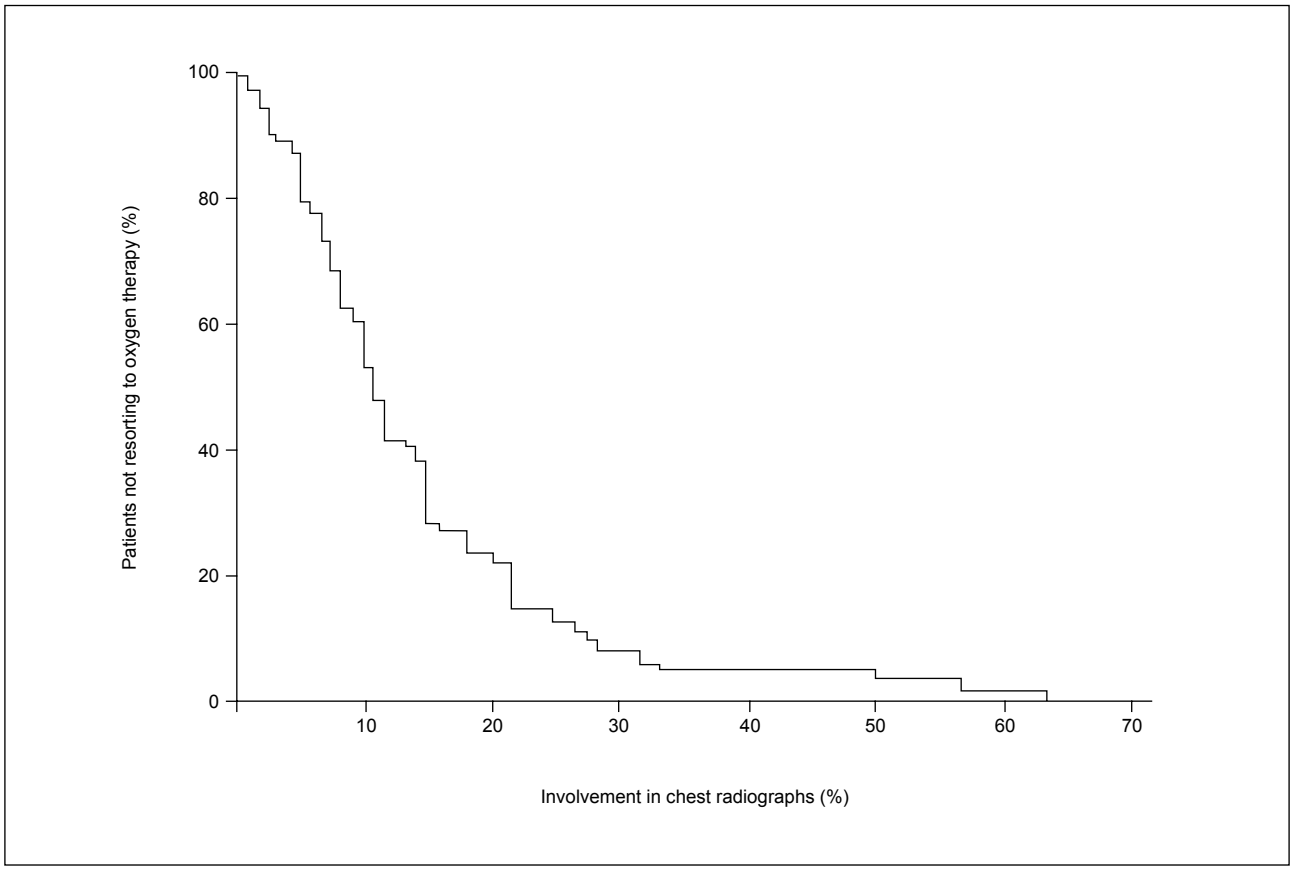

Fig. Kaplan-Meier curve of cumulative % of patients with SARS free of supplementary oxygen requirement versus the extent of consolidation
Even if only a small percentage (10%) of the lung showed consolidation, approximately 50% of such patients received supplementary oxygen[6]

were independent predictors of poor outcome. The radiographic pattern of progression also correlated with clinical outcome. Patients with type-1 pattern seemed to have more favourable outcomes. By contrast, all seven patients with type-4 pattern (progressive deterioration) had adverse clinical outcomes; six died and the seventh was critically ill and had a prolonged ICU stay.[6] Chest radiographs correlate positively with the rate of change of LDH, a marker of tissue damage. The LDH can reflect the extent of lung injury, and both serial chest radiographs and LDH levels are important in the management of SARS. After multivariable analysis, more than one zone being involved in initial chest radiographs was an independent predictor of adverse outcomes even after adjusting for high baseline LDH levels, advanced age, and high neutrophil counts.[6]

Conclusions

In SARS patients, more extensive airspace disease at presentation is an independent predictor of adverse outcome (ICU admission or death).[6] Chest radiography is therefore a useful diagnostic and management tool.

Acknowledgements

This project forms part of a series of studies commissioned by the Food and Health Bureau of the Hong Kong SAR Government and was funded by the Research Fund for the Control of Infectious Diseases. The results of this study have been reported in the following publication:
Hui DS, Wong KT, Antonio GE, et al. Severe acute respiratory syndrome: correlation between clinical outcome and radiologic features. Radiology 2004;233:579-85.

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Super-spreading events of SARS in a hospital setting: who, when, and why?

Key Messages

1. Factors related to the ward environment/administration were important in nosocomial outbreaks of SARS.
2. With the current threat of avian influenza and other respiratory infections such as tuberculosis, hospital wards have to be re-designed and the daily operation reviewed to minimise environmental factors associated with nosocomial infections.

Introduction

It is believed that the SARS coronavirus originated from wild animals and that human-to-human transmission first occurred in Guangdong Province, China. A resident of Guangzhou, who stayed in a Hong Kong hotel in February 2003, was identified as the index case for the spread of SARS to at least five countries.[1] In the resulting epidemic, 1567 and 1755 probable cases occurred in Guangzhou and Hong Kong, respectively.[2]

A super-spreading event (SSE) is defined as a cluster of SARS infections in which one or more individuals infected many more individuals than did an average SARS patient. About 71% and 75% of the infections were attributable to SSEs in Hong Kong and Singapore, respectively.[3] The transmission efficiency of the disease was quite low in the community, with the exception of the Amoy Gardens SSE in Hong Kong.[3] Most SSEs occurred inside hospitals, but the underlying causes have not been well studied. The World Health Organization attributed the SSEs to the lack of stringent infection control measures in hospitals during the early days of the epidemic.[4] A study of four super-spreaders in Beijing found that they were likely to be older, associated with a higher fatality rate and a larger number of close contacts than non–super-spreaders.[5] Other studies focused on the risk factors at the individual level of the secondary cases among health care workers or in-patients, or were simply anecdotal reports based on personal observations and speculations. To better understand why nosocomial outbreaks of SARS occurred and to provide guidance for the prevention of SSEs in the future, we systematically analysed the risk factors associated with nosocomial outbreaks in hospital wards in Guangzhou and Hong Kong through a case-control study.

Aims and objectives

1. To identify SSEs for SARS that occurred in public hospitals in Hong Kong and Guangzhou; and
2. To explore the factors that result in the development of SSEs in a hospital setting.

Methods

Study design and population

A case-control study was designed with individual hospital wards as the units for data collection and analysis. Cases were hospital wards with SSEs for SARS, whereas controls were hospital wards that admitted SARS patients but did not have SSEs. We defined an SSE in a ward as three or more than three new cases occurring within 2 to 10 days after admission of the first patient or an identifiable index case, or within 8 days without any known sources. There is no universally accepted critical (cut-off) number for defining an SSE, but as the basic reproductive number in the community was 2.721, we adopted a more conservative operational definition with a critical number of three or more than three new cases.

Super-spreading events were identified through two means: (1) reports of known nosocomial outbreaks from the infection control units of all hospitals, (2) plotting the date of symptom onset of each case among health care workers and in-patients for each ward to identify clustering, as well as the date of admission of all new cases (including known cases transferred from other wards or hospitals).

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Efforts were made to document evidence of contact between the index case and 'secondary cases'. It was possible that a definite index case could not be identified.

All hospital wards in Guangzhou and the New Territories East Cluster of Hospitals in Hong Kong that admitted at least one case of SARS were included. Paediatric wards were excluded, as the characteristics of SARS in children were quite different from those of adults. Designated wards for treating known SARS patients were also excluded, because of possible multiple contacts with multiple-source cases.

Data collection

Data related to environmental/administrative factors (including physical factors, procedural/situational factors and administrative factors during the 10 days immediately after admission of the index case [for case wards] or the first new case [for control wards and case wards without an identifiable index case]) and host factors (including symptoms, severity/dependency, treatment/intervention and comorbidity of the known index case in a case ward, or the first-admitted case in a control ward) were collected.

Ward managers or nursing officers of the included wards were interviewed in person using a structured questionnaire between September 2004 and November 2005; data were verified using staff rosters. Distances between beds were measured with a measuring tape. Medical records of all SARS patients in the included wards were reviewed to extract information related to the hosts.

Statistical analysis

All data were double-keyed into a pre-designed database and analysed using the SAS software. Univariate analysis was first conducted for each risk factor. Risk factors with a P value of <0.15 were included in a multiple logistic regression analysis using the stepwise approach. This analysis was done separately for environmental/administrative factors and host factors because of the smaller usable numbers of cases and controls related to the host (resulting from unidentified index patients in some case wards or missing data). The odds ratio (OR) and the 95% confidence interval (CI) of various risk factors for the nosocomial outbreak or SSE in a ward were then estimated.

Subgroup analyses by geographic location (Guangzhou and Hong Kong) were also carried out to examine the consistency of risk factors between the two cities. All risk factors selected into any of the separate multivariate models (P<0.15) for environmental/administrative factors and host factors were then included in a combined model using the stepwise approach. As the number of case wards was small and the number of risk factors examined large, some individual risk factors were grouped into composite variables by counting/scoring (the number of positive responses in the group), re-coding (any positive response in the group coded as positive for the composite variable), or ranking according to hierarchy for the statistical analyses. Composite variables by counting or ranking were re-scaled from 0 to 1 in order to equalise their weights in the logistic models. A relatively large alpha error of 0.15 was adopted in the logistic regression analyses in order not to miss potentially important risk factors, as the number of cases included was small. The 95% CI of the OR was used for assessing the statistical significance at the level of P=0.05.

Results

In Guangzhou, 87 wards in 21 hospitals were included. In Hong Kong, 40 wards in five hospitals were enrolled. Two paediatric wards were excluded. We failed to obtain relevant information in only one ward in Guangzhou and two wards in Hong Kong.

Of the 86 wards in Guangzhou, 35 (41%) were classified as case wards, 26 (74%) of which were identified with an index patient. Of the 38 wards in Hong Kong, 13 (34%) were classified as case wards, five (39%) of which were identified with an index patient. The male-to-female ratio was 1.4:1 among index patients and 1.1:1 among first-admitted patients in control wards. The index patients in the case wards were slightly older (mean age, 51 vs 49 years) and had a longer lag time from symptom onset to hospital admission (8 vs 6 days) than the first-admitted patients in the control wards.

In the univariate analysis, environmental/administrative factors that significantly associated with an SSE included the minimum distance between beds being ≤1 m, the lack of washing/changing facilities for staff, exhaust fan never used, the use of a high flow rate O_2 mask, performance of resuscitation, staff working with symptoms, and high workload (with a patient/health care workers ratio of >2). Contamination events and infection-control training were not significant factors (P=0.05-0.15). Significant host factors included pulmonary congestion, resorting to oxygen therapy, higher severity of disease, the use of nebuliser, and the use of bi-level positive airway pressure ventilation (BIPAP). Respiratory symptoms (cough and phlegm), systemic symptoms (myalgia, chills, rigor, malaise, headache and dizziness) and dependency (for activities of daily living and behaviour changes) were not significant factors (P=0.05-0.15).

In the analysis combining data from Guangzhou and Hong Kong, three factors were significant: the minimum distance between beds being ≤1 m (OR=3.36), the lack of washing/changing facilities for staff (OR=0.21), and staff working with symptoms (OR=5.50). Performance of resuscitation was not a significant factor (P=0.10). The minimum distance between beds being ≤1 m was the only factor present in both the Guangzhou and Hong Kong models, though for the latter, it was only of borderline significance (P=0.07).

In the multiple logistic regression analysis for host factors, the use of oxygen therapy and systemic symptoms were significant in the Guangzhou model but not in the Hong Kong model. In the analysis for combined data, only resorting to oxygen therapy was significant (OR=3.59). The use of BIPAP had a P value of 0.06.

Four environmental/administrative factors and two host factors resulted from the final model combining data from Guangzhou and Hong Kong were significant: the minimum distance between beds being ≤1 m (OR=6.94, 95% CI=1.68-28.75), the lack of washing/changing facilities for staff (OR=0.12, 95% CI=0.02-0.97), performance of resuscitation (OR=3.81, 95% CI=1.04-13.87), staff working with symptoms (OR=10.55, 95% CI=2.28-48.87), resorting to oxygen therapy (OR=4.30, 95% CI=1.00-18.43), and the use of BIPAP (OR=11.82, 95% CI=1.97-70.80). Two environmental/administrative factors emerged consistently in the three models: the minimum distance between beds being ≤1 m and staff working with symptoms. Two environmental factors (the lack of washing/changing facilities for staff and performance of resuscitation) did not emerge in the separate models for Guangzhou and Hong Kong, but were significant in the overall model. Exhaust fan never used and systemic symptoms emerged only in the model for Guangzhou (P=0.05-0.15), but not Hong Kong or the overall model.

Sensitivity analysis was conducted by varying the critical number for defining an SSE. With a cut-off value of four cases of nosocomial spread of SARS in a single ward, five factors emerged in the final combined model, including three significant factors in the model with a cut-off value of three cases (minimum distance between beds being ≤1 m, staff working with symptoms, and resorting to oxygen therapy). Systemic symptoms in the host became a significant risk factor and the use of a high flow rate O_2 mask in the ward was included in the model (P=0.12). Using a cut-off value of five cases, five significant factors were present in the final combined model: minimum distance between beds being ≤1 m, staff working with symptoms, resorting to oxygen therapy, systemic symptoms, and the use of a high flow rate O_2 mask in the ward.

Discussion

We analysed risk factors associated with nosocomial outbreak of SARS in a systematic manner, using an analytic epidemiological design. Significant environmental risk factors associated with the occurrence of SSE (clustering of three or more cases) included minimum distance between beds being ≤1 m and performance of resuscitation. The use of BIPAP and oxygen therapy were significant risk factors related to the host. Administratively, allowing staff with symptoms to work also increased the risk. Providing adequate washing/changing facilities for staff was protective. Sensitivity testing by applying more stringent cut-off points (four or five clustered cases) suggested that our results were quite robust, with three significant risk factors being identified consistently: minimum distance between beds being ≤1 m, staff working with symptoms, and host resorting to oxygen therapy.

Environmental and administrative factors were important in the prevention of nosocomial outbreaks of SARS. These factors have also been identified as risks for nosocomial spread of other respiratory infectious diseases and they should be rectifiable. Inadequate bed spacing and overcrowding in hospital wards increases the risk of nosocomial infection outbreaks.[6-9] Unfortunately, it is a usual practice to increase the number of hospital beds to meet with the increasing demand, especially during an epidemic. This practice is against the original design of the hospital ward and infection control policy. When the distance between beds is reduced, droplet can spread from one patient to the adjacent patients and ventilation (natural or mechanical) can also be jeopardised. A place for medical treatment and care then becomes a hazardous environment, both for the patients and staff.

Staff working with symptoms could spread SARS in hospital wards and this risk factor is consistently found in all three models in the current analysis. The SARS coronavirus load in patients is highest in the first week of the infection and the patient is most contagious when febrile.[10] Therefore, staff working with symptoms might account for some nosocomial outbreaks where no index patients could be identified.

Provision of washing/changing facilities in hospital ward for staff helped to reduce the risk of nosocomial outbreak. This also suggested that health care workers could act as passive carrier of the SARS coronavirus leading to nosocomial transmission of the infection.

The use of oxygen and BIPAP in patients with infectious respiratory diseases has been a subject for debate since the SARS outbreak. The high flow rate of oxygen/air and/or the positive pressure resulting from such treatment procedures might accentuate the spread of potentially infectious exhaled/expelled air from such patients.[11] Exhaled air from a mask can travel to 0.4 m on each side of the patient.[12] In the present study, the use of oxygen therapy and BIPAP both imposed a significant risk for nosocomial spread of SARS in the model with combined data from Guangzhou and Hong Kong with a cut-off value of three cases and the use of oxygen therapy also significantly increased the risk of nosocomial outbreaks in models with higher cut-off values. We did not have enough detail about the oxygen therapy modalities to the index cases to allow a more refined analysis regarding the types of mask/cannula and the flow rate of oxygen supply. Proper capturing (enclosure/containment/local exhaust) and filtering (high efficiency particulate air filter) of exhaled/expelled air should be implemented if oxygen therapy and BIPAP must be used on clinical grounds. The mechanical manoeuvres associated with resuscitation

can potentially generate large amounts of aerosols that are infectious, especially during intubation of the airway and manual bagging to support ventilation of the patient. More thought should be given to redesigning the procedures, by engineering or administrative means, to achieve effective containment of any possible contamination arising from the resuscitation process.[13]

Higher occurrence of systemic symptoms in the index or first case emerged as a significant risk factor when SSE was defined by clusters of 4+ or 5+ cases. It is not known if this could be related to a higher viral load. Higher viral loads had been reported to be associated with oxygen desaturation, diarrhoea, hepatic dysfunction, mechanical ventilation and death. However, clear relationships with systemic symptoms have not been reported.

Although the participation rate of this study was very high (97.6%, 124/127 of all eligible wards), the study was confined to two centres in southern China and the applicability to other countries with different hospital practices was not known. Nonetheless, our study provided evidence on risk factors for SSE in the hospital setting. The interviews were carried out more than 1 year after the outbreak and recall inaccuracies might exist. Site inspections and physical measurements were performed on various environmental risk factors and documents and staff roosters reviewed. Information bias should have been substantially reduced. Nonetheless, all host factors were extracted from original medical records and may be objective. Another intrinsic weakness was the lack of statistical power due to the small number of case wards, especially in subgroup analysis for Hong Kong. Hence, the contribution of certain risk factors (such as type of ventilation in ward, lack of appropriate personal protective equipment and infection-control training) could not be ruled out entirely. A larger international collaboration may help solve this problem. The consistent results in different subgroup analyses in Hong Kong and Guangzhou provide indirect support that our results are in general valid. Environmental/administrative factors were more important than host factors. Other than the presence of systemic symptoms (in analyses with more restrictive definitions for SSE), the two host factors identified (ie the use of oxygen therapy and BIPAP) pertained more to environmental contamination than patient characteristics. In other words, this study managed to characterise the super-spreading environment more than the so-called super-spreaders.

Conclusions

With the current threat of avian influenza and other respiratory infections such as tuberculosis, hospital wards have to be re-designed and the daily operation reviewed to minimise environmental factors associated with nosocomial infections. Adequate spacing between beds and provision of washing/changing facilities for staff are important. Staff with symptoms of respiratory infections should refrain from working in the wards. Adequate protective devices should be provided. More work should be done on the safe use of oxygen therapy and/or ventilatory support in patients with respiratory infections.

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A comparative study of the stigma associated with infectious diseases (SARS, AIDS, TB)

Key Messages

1. Quantitative and qualitative approaches were used to elucidate the psychosocial processes operating in SARS, HIV/AIDS, and tuberculosis.
2. The impact of stigma was examined from three perspectives: (1) the general public (public stigma), (2) target individuals afflicted with stigma (self-stigma), and (3) affiliates of the target individuals (affiliate stigma).
3. Three dimensions of stigma were assessed: (1) cognition (stereotypes and beliefs about the target), (2) affect (prejudicial attitudes and feelings toward the target), and (3) behaviour (discrimination toward the target).

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Introduction

Stigma can be defined as an attribute linking a person to a set of undesirable characteristics that may lead to prejudice and discrimination. Infectious diseases are considered stigmatising.[1] Stigma causes psychological suffering (eg shame and isolation) in afflicted individuals and families, compromises efforts to provide effective health care, and fosters discrimination in societies. Stigma may also directly affect the course and outcome of the stigmatised medical condition, by increasing stress or decreasing access to effective treatment.[2] For example, individuals with the stigmatising condition may delay treatment for fear of being labelled with the condition, or they may avoid treatment because the treatment setting has been made so undesirable that they may be discouraged from seeking help. In HIV/AIDS, stigma has been shown to be associated with self-esteem and depression.[3] Given the impact of stigma on health, research is needed to guide desirable public health interventions for the reduction of stigma. This can be done by comparing the features of stigma between different diseases, understanding the phenomenon of stigma and its psychosocial correlates within particular sociocultural contexts, documenting the burden of stigma on those afflicted as well as their associates, and evaluating the process of stigmatisation over time and in response to the course of the diseases, interventions, and social change.[4]

Limited research has been done on the stigma associated with infectious diseases among Chinese, and work done in other societies may not be extrapolated to the Chinese population because of cultural differences. Local studies examining attitudes toward people with mental illness[5-9] and with HIV/AIDS[10,11] tend to be descriptive in nature and have focused on public perceptions. Small-scale ethnographic case studies have been carried out among SARS sufferers. Media reports suggest that SARS and HIV are both stigmatising conditions, which result in self-barricading and social rejection of those in contact with SARS sufferers, and hiding of information by those who have contracted HIV from contaminated blood products.[12] There have also been anecdotal reports of people losing their jobs because they have family member(s) with SARS. In view of the rapidity of spread and the serious consequences, both in terms of mortality and subsequent protracted physical and psychological morbidity, observed during the recent SARS epidemic, considerable stigma may be attached to SARS. To assess the extent of SARS stigma, other stigmatising infectious diseases (ie HIV/AIDS, tuberculosis [TB]) may be used as a benchmark for evaluating the phenomena. Both the differences and commonalities observed across these conditions can enlighten researchers about psychosocial sequelae such as reduced self-esteem, social disadvantages, or changes in health-seeking behaviours. All of these have implications for the design of relevant public health interventions. Furthermore, stigma must be understood from multiple perspectives (self-stigma, affiliate stigma, public stigma) so that interventions aiming to reduce stigma can target each condition from multiple levels. Responses by the general public, potential stigmatisation of associates of the affected person (family members, friends, healthcare workers), and internalisation of stigma by the affected persons are all important processes that stigma reduction programmes need to address. Measures may include legal action, changes to the health care system, support from the public, professionals, and family members, and self-care and understanding of those affected.

Aims and objectives

1. Explore the sociocultural and psychological underpinnings of public, self-, and affiliate stigma;
2. Identify social-cognitive processes that are germane to the adoption and maintenance of stigma;
3. Examine the effects of stigma by accounting for individuals' well-being and social opportunities; and
4. Compare the magnitude of stigmatisation and its related outcomes among SARS, HIV/AIDS, and TB.

Methods

Qualitative study

A total of 90 participants were interviewed for 19 focus groups: 17 for public stigma, 22 survivors/patients and 11 caregivers for SARS, six survivors/patients and eight caregivers (including non-government organisation service providers and health care professionals) for HIV/AIDS, and 20 survivors/patients and six caregivers for TB.

Quantitative study

Public stigma

A telephone survey was conducted between September and October 2004, to assess levels of public stigma related to SARS, HIV/AIDS and TB among Hong Kong residents. Telephone numbers were drawn randomly from a pool of seed numbers based on the most recent residential telephone directories, which contained almost all residential telephone numbers in Hong Kong. To capture unlisted numbers, the last two digits of the number selected were deleted and replaced by two random numbers generated by computer. If the household could not be reached, two more follow-up calls were made at different hours. The interviews were conducted between 6 and 10 pm on weekdays and 2 to 9 pm on Saturdays to avoid under-sampling of students and employed individuals. One eligible household member aged 18 to 65 years whose birthday was the closest to the interview date was invited to participate in each residential unit. The selected participant was interviewed about one of the above three infectious diseases, based on random assignment, and the interview was conducted in Cantonese. A total of 3011 participants took part in the interview, of which 1007, 1001, and 1003 participants were questioned about HIV/AIDS, SARS, and TB, respectively. The response rate, defined as the number of complete interviews divided by the total number of households containing an eligible person contacted, was 45.5%, 47.3%, and 50% for HIV/AIDS, SARS, and TB, respectively.

Self-stigma

Patients with one of the three health conditions were interviewed twice in a 6-month interval. They were recruited using the following methods: (1) recovered SARS patients identified from a list provided by the Hospital Authority: first-wave data from 147 ex-SARS patients and second-wave data from 106 ex-SARS patients were collected. (2) People with HIV/AIDS attending government AIDS clinics and major non-government organisations such as the Hong Kong AIDS Foundation: first-wave data from 150 people with HIV/AIDS and second-wave data from 119 people with HIV/AIDS were collected. (3) People with TB from hospitals (Tai Po Hospital) as well as chest clinics (Yuen Chau Kok): first-wave data from 148 people with TB and second-wave data from 85 people with TB were collected.

Trained interviewers sought informed consent from the participants by explaining the purpose of the study, confidentiality of the data, and rights of the participants. Interviewers conducted the interview at a location preferred by the participants and on completion of the questionnaires, participants were paid HK$50 or a coupon equivalent as compensation for their time.

Affiliate stigma

Patients who participated in the study and agreed that their primary caregivers may be contacted for collateral information were asked to identify, at most, three family members who regularly provide care for them. Identified affiliates were contacted by a research assistant and asked to participate in the study to explore their experience of caring for individuals with SARS, HIV/AIDS or TB. The caregivers were interviewed twice in a 6-month interval. The following data were collected: (1) SARS caregivers: first-wave data from 74 ex-SARS caregivers and second-wave data from 64 ex-SARS caregivers were collected. (2) HIV/AIDS caregiver: first-wave data from 7 HIV/AIDS caregivers were collected and no second wave data collection due to small sample size in the first wave. (3) TB caregivers: first-wave data from 57 TB caregivers were collected and no second wave data collection due to small sample size in the first wave.

The interviews were conducted at locations selected by the participants who were paid HK$50 or a coupon equivalent to compensate for their time.

Results

Qualitative study

Public stigma focus group findings: physical and psychological avoidance was the most common reactions toward people known to have infectious diseases (SARS, HIV/AIDS, and TB). Most participants were knowledgeable about the modes of transmission of the infectious diseases under study. Attribution of stigma was somewhat different between SARS/TB and AIDS. This might be because people with SARS/TB were not generally seen as being responsible for contracting the disease whereas HIV infection is viewed as a self-inflicted, avoidable consequence.

The SARS focus group findings: quite a number of participants reported apathetic attitudes and avoidant behaviours from doctors during their in-patient stay. They also reported avoidance from family, friends, colleagues, and neighbours after recovery. Many perceived barriers to

service access and employment after recovery. Emotional disturbances were reported quite frequently, including worries, anxiety, and a sense of helplessness.

The HIV/AIDS focus group findings: compared with the two other groups, it was much more common for people with HIV/AIDS to maintain secrecy as they regarded AIDS a highly stigmatised disease. All agreed that HIV-related stigma is rooted in biased publicity about the nature of the disease, judging it from a moralistic point of view. Actual and anticipated rejection by others predisposed people with HIV/AIDS to feel self-hatred, humiliated and to withdraw.

The TB focus group findings: no participants reported stigma in medical settings, though interpersonal avoidance by family members, friends/relatives, and neighbours is still common. Quite a number of participants believed public fear of TB has been reduced by better knowledge about the disease.

Quantitative study

For the telephone survey about public stigma toward SARS, HIV/AIDS, or TB, path-constrained structural equation models were compared. The attribution model (internal controllability, responsibility and blame) was predictive of an increased level of self-stigma (CFI=0.92, RMSEA=0.04). The MANOVA results indicated that the high- and low-stigma groups showed significant differences in attitudes to policy across the three diseases. The low-SARS stigma group expressed more favourable attitudes toward government policies on prevention, public education, research, and anti-discrimination than their high-SARS stigma counterparts. As for HIV/AIDS, significant differences in attitudes were found only in attitudes to policies on prevention, public education, and anti-discrimination. Finally, differences in attitudes between low- and high-TB stigma groups were found only in attitudes to policies on prevention, public education, and research.

Data from first-wave SARS survivors and their caregivers were analysed to elicit the relationship between their self-stigma. Caregiver strain was a significant mediator between affiliate self-stigma and survivor self-stigma among 51 dyads.

First- and second-wave people with HIV/AIDS findings: structural equation modelling was used to analyse the data. The results indicated (CFI=0.97, RMSEA=0.08) that the attribution model (personal responsibility, stability, and personal controllability) was not predictive of self-stigma. A higher level of self-stigma led to a decreased level of social support, and eventually a higher level of mental distress.

In terms of medication adherence for people with HIV/AIDS, using the conventional adherence rate, only 12 (11.8%) of participants reported having missed/altered medication in the past 4 days. However, using a more comprehensive assessment, only 27 (26.5%) of participants were classified as adherers. Intentional non-adherers had worse mental health, higher level of self-stigma, and higher score in avoidant coping than adherers and unintentional non-adherers. They also scored higher in physical symptoms than adherers.

Discussion

The telephone survey results indicate that public stigma is greatest toward HIV/AIDS, followed by TB then SARS. Using multi-sample structural equation modelling, the attribution model with internal controllability, personal responsibility, and blame were found to be applicable across the three diseases for explaining stigma. Knowledge about the disease had no significant effect on stigma. Participants with less stigmatising views had significantly more favourable attitudes toward government policies related to the diseases.

Data from the 119 people with HIV/AIDS indicated that although the linkage between the attributions of control, responsibility, and blame was confirmed, the relationship of blame to self-stigma was not significant. Self-stigma was found to dampen social support and lead to psychological distress half a year later.

Data from 143 SARS survivors indicated that self-care self-efficacy completely mediated the effects of perceived medical staff support and perceived family/friends support on mental health status.

Regression analyses on data from 51 dyads of SARS survivors and their caregivers, indicated that affiliate self-stigma served as a partial mediator between patient self-stigma and caregiver strain.

Conclusions

This study is an important attempt to understand the attributional mechanisms of stigma toward infectious diseases. It challenges the adequacy of attributional factors as a means of understanding self-stigmatisation and demonstrates the impact of stigma on psychological adjustment among people with HIV/AIDS. It is also the first attempt to understand long-term psychological adjustment in SARS survivors. These findings may be applicable to other infectious disease outbreaks because they inform about psychosocial factors that may be important to long-term recovery. Caregivers for patients with higher self-stigma are at risk of greater internalisation of stigma and caregiver strain.

Stigma reduction and promotion of public awareness should focus not only on knowledge but also cognitive representations of illness and interpersonal contact to alleviate stigma. Along with providing psycho-education and information about treatment and medication, family-based interventions should focus on the self-stigma imposed

on patients and caregivers.

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Role of cytokines and chemokines in severe and complicated influenza infections

Key Messages

1. Exuberant systemic cytokine and chemokine responses are present in naturally occurring influenza infection. These are associated with more severe clinical manifestations, and can be linked to uncontrolled viral replication and signalling molecule hyperactivation.
2. Early antiviral treatment may suppress these harmful cytokine/chemokine responses.

Introduction

Severe seasonal influenza accounts for more than 226 000 hospital admissions in the United States annually.[1] In Hong Kong it is responsible for 15 to 50 hospital admissions per 10 000 elderly people who may suffer from various complications including pneumonia, bronchitis, exacerbations of chronic pulmonary diseases, and even heart attacks and strokes.[2] The mortality rate in these hospitalised patients is high, ranging from 4 to 29%, but few clinical studies have investigated the immunopathogenesis of severe influenza infection.[3,4] In mild, uncomplicated H1N1 infections (either experimental or naturally occurring), plasma and nasal IL-6 concentrations have been found to be elevated, and to correlate with both viral titres and systemic and respiratory symptoms. Significant releases of IL-8, TNF-α, IFN-α/γ, and IL-10 into nasal fluid or peripheral blood have also been detected. Nonetheless, the number of cytokines that have been studied is limited, and the question whether certain chemokines (eg IP-10) play a role in naturally occurring influenza infections remains unanswered.[5,6] Studies on H5N1 (avian influenza) disease have suggested that a 'cytokine storm' (eg IL-6, IL-8, IP-10, MIG, MCP-1) occurs due to uncontrolled viral replication and may be responsible for its clinical manifestations and poor outcomes.[7-11] Hyperactivation of certain signalling molecules such as phospho-p38 mitogen-activated protein kinase (MAPK) has been linked to the production of this 'cytokine storm'. With the imminent threat of another influenza pandemic, studies on the immunopathogenesis of severe influenza enable hypothesis generation for developing novel therapeutic approaches.

In this study, we aimed to detect systemic cytokine and chemokine responses in naturally occurring, severe human influenza infection, and to examine their correlations with clinical disease severity, level of viral replication, and signalling molecule activation. We found an intense pro-inflammatory and Th1 cytokine/chemokine response in severe infection that is associated with uncontrolled viral replication.

Aims and objectives

1. To study the changes of various cytokines/chemokines among hospitalised acute influenza-infected patients;
2. To compare pre-treatment cytokine/chemokine profiles with their convalescent phase profiles; and
3. To correlate these with age, co-morbidity, symptom severity, complications, and viral loads.

Methods

Patients

A prospective observational study on consecutive laboratory-confirmed adult (age ≥18 years) influenza patients admitted to the Prince of Wales Hospital during a peak flu season in 2006 (1 February to 31 July 2006) was conducted. Patients were diagnosed and managed according to the standard protocol. They were admitted to designated medical wards and placed on droplet precautions when they had developed potentially serious medical conditions, or when the

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exacerbation of their chronic underlying illnesses or severe symptoms were considered unmanageable at home.[12] A nasopharyngeal aspirate (NPA) was taken to test for influenza A and B infections using immunofluorescence assays (IFA; the panel also included parainfluenza, respiratory syncytial virus [RSV] and adenovirus), together with blood and sputum cultures to exclude secondary infections. Patients were recruited once their NPA/IFA tested positive for influenza A. After obtaining informed consent, 12 mL ethylenediaminetetraacetic acid venous blood samples (the acute phase samples) were taken from the patients on the same day as antiviral treatment commenced. Patients were followed up 7 to 10 days after the acute symptoms had subsided, and the blood sampling was repeated (convalescent phase samples). There was no medical intervention. The patients were managed and discharged according to their physicians' usual practice. In particular, antiviral treatment choices were not affected by their decision to participate. The institutional review board of the Chinese University of Hong Kong and the Hospital Authority of Hong Kong approved this study.

Virological investigations

The NPA samples collected were used for immunofluorescence staining, virus isolation and influenza viral RNA quantification. A commercial IFA for influenza A and B (Chemicon International, CA, US) was used to make the initial diagnosis of influenza infection. Influenza virus isolation was conducted using MDCK cells, and cell monolayers were examined daily for any cytopathic effect. After 14 days of incubation, the growth of influenza was examined using haemadsorption, and confirmed by immunofluorescence staining using influenza group-specific antibodies (Chemicon International, CA, US), which identified the isolate as either influenza A or B. Influenza A isolates were further differentiated into H1 and H3 subtypes. To estimate the amount of influenza A RNA in the upper respiratory tract, total RNA was extracted from the supernatants of the NPA specimen using a QIAamp Viral RNA extraction kit (Qiagen, Hilden, Germany). The resulting complementary DNA products (cDNAs) were subjected immediately to a real-time polymerase chain reaction (PCR). The primers used were 5'-AAG ACC AAT CCT GTC ACC TCT GA-3' (forward) and 5'-CAA AGC GTC TAC GCT GCA GTC C-3' (reverse), which amplified a 74-base pair fragment in the M gene of influenza A. These primers were designed to detect influenza A RNA originating from the circulating H1N1 and H3N2 viruses. The real-time PCRs were carried out in a 96-well microtitre plate using ABI7900 (Applied Biosystems).[13]

Measurement of plasma cytokines and chemokines

The ethylenediaminetetraacetic acid blood samples were immersed in ice and transported immediately for processing. Plasma was separated by centrifugation (2000 xg for 10 min) at 4°C and stored in 300 μL aliquots at -70 °C until analysis. Inflammatory cytokines IL-1β, IL-6, IL-10, IL-12p70, tumour necrosis factor (TNF)–α; and chemokine CXCL8/IL-8, monokine induced by IFN-γ (CXCL9/MIG), IFN-γ-inducible protein–10 (CXCL10/IP-10), monocyte chemoattractant protein–1 (CCL2/MCP-1), and regulated upon activation normal T cell-expressed and secreted (CCL5/RANTES) were measured simultaneously using bead-based multiplex flow cytometry with human inflammatory cytokine and chemokine cytometric bead array (CBA) reagents, respectively (four-colour FACSCalibur flow cytometer, BD Biosciences Corp, San Jose [CA], US). The choice of cytokines and chemokines investigated was based on the results of previous studies on seasonal and avian influenza infection. The CBA uses different bead populations with distinct fluorescence intensities that have been coated with capturing antibodies specific for different cytokines or chemokines. After incubation with 50 μL of plasma, the beads that had captured cytokines/chemokines were mixed with phycoerythrin-conjugated detection antibodies to form sandwich complexes. Fluorescence flow cytometry of the beads provide simultaneous quantification of a panel of cytokines and chemokines. Plasma concentrations of IFN-γ were quantified using an ELISA kit (R&D Systems, MN, USA). The assay sensitivities of IFN-γ, IL-1β, IL-6, IL-10, IL-12p70, TNF-α, CXCL8/IL8, CCL5/RANTES, CCL2/MCP-1, CXCL10/IP-10 and CXCL9/MIG were 7.1, 2.5, 3.3, 3.7, 1.9, 7.2, 0.2, 1.0, 2.7, 2.8, 2.5 pg/mL, respectively, as previously published. The coefficients of variation were all <10%. Their respective reference ranges (RF) were derived from the measurement of more than 100 healthy controls as previously described.[14]

Analysis of intracellular signalling molecules

Differential activations of selected intracellular signalling molecules, including phospho-p38 MAPK, phospho-extracellular signal-regulated protein kinase (ERK), and pJNK in the T-lymphocytes (both CD4+ and CD8+ cells) were studied in the first 12 patients and compared to 14 age- and sex-matched healthy controls. In brief, PBMC was separated; fluorochrome-conjugated antihuman MAPK or mouse IgG isotopic antibody was added, incubated with the cells, and subjected to flow cytometric analysis using CD4/CD8 cell gating. Results were expressed as mean fluorescence intensity (MFI).[15]

Results

Thirty-nine patients with H1N1 infection were studied. Their mean age was 57±21 years and 56% had underlying medical conditions. Over 70% of patients developed influenza-related complications, which included pneumonia, bronchitis, exacerbation of chronic obstructive pulmonary disease/asthma, acute cardiovascular or cerebrovascular events, encephalopathy and syncope. Nearly half of the patients required supplemental oxygen therapy. Over 35% of these patients had prolonged hospitalisation for more than 5 days. No patient in this cohort died.

Cytokine/chemokine concentrations (pg/mL) in plasma samples obtained during the acute phase were compared to

convalescent phase concentrations. We found significant increases in IL-6 (10.6 [range, 4.2-18.4] vs 2.9 [range, 1.6-7.0]; RF, <3.1), IL-8 (5.4 [range, 2.5-8.7] vs 2.1 [range, 0.2-3.5]; RF, <5.0), IP-10 (7043.0 [range, 4025.1-12381.1] vs 1423.6 [range, 931.8-1634.8]; RF, 202-1480), MIG (992.1 [range, 499.1-1992.3] vs 431.7 [range, 198.4-792.9]; RF, 48-482) and MCP-1 (76.5 [range, 49.5-97.0] vs 56.6 [range, 41.2-84.8]; RF, 10.0-57.0) during the acute illness (overall 2-5 fold increase; Wilcoxon signed-rank test, P<0.01). The highest cytokine/chemokine concentrations were noted on symptom onset days 3 to 4. During the convalescent phase, all these cytokine/chemokine levels dropped, but the RANTES concentration increased (1851.8 [range, 667.4-4774.3] vs 4742.8 [range, 2767.5-5169.7]; P<0.05). The acute phase samples were collected 2.8±1.2 days after symptom onset. No significant changes in IFN-γ, IL-1β, IL-10, IL-12p70 and TNF-α concentrations were detected.

Viral RNA concentrations in the upper respiratory tract were found to correlate significantly with IL-6 (Spearman's ρ=+0.41, P=0.015), IL-8 (ρ=+0.49, P=0.003), IP-10 (ρ=+0.54, P=0.001), MIG (ρ=+0.46, P=0.005) and MCP-1 (ρ=+0.43, P=0.011).

Hypercytokinaemia (eg IL-6, IL-8, MIG, MCP-1) occurred in patients who were of advanced age, had major comorbidities, and developed cardiac/respiratory complications (Mann-Whitney *U* test, all P<0.05). These patients were also found to have higher viral loads (RNA concentration) in their respiratory tracts (P<0.05). In a multivariate logistic regression analysis, it was found that an elevated IL-6 plasma concentration was independently associated with prolonged hospitalisation of >5 days, adjusted for age, co-morbidity and viral load (odds ratio [OR], 14.4; P=0.020).

Hypercytokinaemia was linked to hyperactivation of intracellular signalling molecules. In CD4+ T-helper cells, the expression of p38-MAPK was enhanced (ie higher MFI) and phospho-ERK was suppressed (Mann-Whitney *U* test, P<0.05); in CD8+ T-cells, both phospho-ERK and pJNK were noted to be suppressed, compared with healthy controls (Mann-Whitney *U* test, P<0.05). Expression of p38-MAPK was found to be associated with elevated IP-10 (ρ=+0.78, P=0.004), MCP-1 (ρ=+0.70, P=0.016), and MIG (ρ=+0.57, P=0.066) concentrations.

Discussion

Exuberant systemic cytokine and chemokine responses are present in naturally occurring influenza infection. These are associated with more severe clinical manifestations, and can be linked to uncontrolled viral replication and signalling molecule hyperactivation. It is possible that early antiviral treatment can suppress these harmful cytokine/chemokine responses. Our findings add to the existing knowledge about influenza immunopathogenesis, and enable hypothesis generation for the development of novel therapeutic approaches against severe influenza infection.

These findings are consistent with earlier in vitro and in vivo cytokine studies on uncomplicated influenza. In mild human H1N1 infection, local (nasal) and systemic (peripheral blood) cytokine responses, such as elevated IL-6 and IL-8 levels, can be detected, and these correlate with symptom severity.[10] In severe, complicated naturally occurring influenza infection, chemokines such as IP-10, MIG and MCP-1 are detectable and have clinical correlations, along with the established influenza chemokines IL-6 and IL-8. IL-6 is a key pro-inflammatory cytokine responsible for fever and influenza symptom formation.[11] It has been associated with various influenza-related complications, including encephalopathy and cardio-respiratory events. Elevated IL-6 is an independent factor associated with prolonged, severe illnesses in hospitalised patients. The exact immunopathogenetic mechanisms and symptom correlations of IL-8 (a neutrophil chemoattractant, implicated in the pathogenesis of ARDS), IP-10 (a chemoattractant for monocytes/macrophages and Th1 cells, which acts as a major systemic inflammatory mediator by activating cell-mediated immunity) and MIG (an indicator of activation of the Th-1 pathway) in severe human influenza infection are less clear and require further study. The cytokine profile observed in naturally occurring influenza is quite similar to that observed in avian influenza (H5N1) disease. In those cases, elevated plasma concentrations of IL-6, IL-8, IP-10 and MIG are seen, but the profile includes other cytokines/chemokines such as MCP-1, IL-10, TNF-α and IFN-α/γ. In addition, systemic concentrations of these cytokines are much higher than those seen in seasonal influenza as reported in this study. The H5N1 virus is known to be a more potent inducer of cytokines than H1N1 or H3N2, which is possibly related to its different internal gene constellation (eg the NS1 gene).[7-9]

Our findings also help to explain the severe symptoms in hospitalised influenza patients.[12] Exuberant cytokine/chemokine responses are present in high-risk patients who are of advanced age and have underlying co-morbid illnesses. Their cytokine levels are much higher than those described for younger patients with uncomplicated influenza. It is likely that impaired host-defence in the high-risk patients have led to more active viral replication (higher viral loads), which in turn induces a more intense cytokine response. Since a positive correlation between virus replication and hypercytokinaemia can be demonstrated, it is possible that early effective suppression of viral replication by antiviral treatment (eg oseltamivir, zanamivir) may result in attenuation of these harmful inflammatory responses.

Our novel finding of in vivo activation of intracellular signalling molecules in acute influenza infection may deserve further investigation. It has been shown that p38-MAPK can induce cytokine expression and apoptosis in experimental influenza models. This molecule is activated in T-lymphocytes in naturally occurring infection, and

its intensity of expression correlates with a hyper-Th1 cytokine response. These findings provide a basis for further investigation of novel therapeutic approaches for treating severe influenza, such as through modulation of cytokines and signalling molecules.[5,6]

Conclusions

Exuberant systemic cytokine and chemokine responses are present in naturally occurring, severe influenza infection, and can be linked to uncontrolled viral replication and signalling molecule hyperactivation. Our findings add to existing knowledge concerning the immunopathogenesis of severe influenza, and enable hypothesis generation for the development of novel therapeutic approaches. This study has broadened our understanding of the immunopathogenesis of severe influenza infection, and has important implications for its clinical management. Hypercytokinaemia occurs in high-risk patients and is related to uncontrolled viral replication. Early, effective viral suppression may result in the attenuation of these harmful cytokine responses and efforts should be made to achieve this goal. Further investigation into the immunopathogenesis of influenza is warranted as this may enable the development of new therapeutic approaches.

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Serum total glycosylation profiling for non-invasive diagnosis of liver cirrhosis in people with chronic hepatitis B

Key Messages

1. A high-throughput assay for quantitative profiling of N-glycans attached to serum glycoproteins has been established.
2. A panel of serum N-glycans were identified as potential biomarkers for diagnosing liver cirrhosis and liver fibrosis.
3. Four glycan peaks of 1341.5, 1829.7, 1933.3, and 2130.3 m/z were all able to detect liver fibrosis and cirrhosis with 85% accuracy.

Introduction

The persistent hepatic inflammation caused by chronic hepatitis B (CHB) infection leads to progressive liver fibrosis, and eventually, liver cirrhosis. Both liver fibrosis and cirrhosis are reversible if treated early. Knowledge of the stage of liver fibrosis is essential for prognostication and decisions about anti-viral treatment.[1,2] Liver biopsy is the gold standard for assessing liver fibrosis based on histological scoring systems.[3] A liver biopsy assessment is recommended whenever anti-viral treatment is considered,[4] but this is an uncomfortable and sometimes risky procedure, so is not suitable for the routine follow-up of CHB patients. Therefore, serum markers that can reliably detect liver cirrhosis are needed, but those currently available are not sufficiently sensitive for effective detection of liver cirrhosis.

In chronic hepatitis C (CHC) infection, serum markers have been used to predict liver fibrosis. It has been suggested that algorithms based on biochemical and haematological markers can correlate with liver fibrosis.[5,6] A commercially available test (FibroTest, BioPredictive SAS, Paris, France) based on a panel of serum protein markers related to liver fibrosis has been developed.[7] Serum-based assays can be used to assess and monitor liver fibrosis in CHC, with area under the 'receiver operator characteristics' curve being 80 to 90%.

In CHB, similar models based on serum biochemical markers have only achieved moderate correlation with liver fibrosis, and show about 50% sensitivity for detecting significant fibrosis.[8] These less encouraging results may be related to CHB's more complicated natural history, as it is characterised by intermittent exacerbations with different disease phases related to the HBeAg status, whereas CHC is generally an indolent, progressive disease.[9]

Considerable evidence indicates that the N-linked carbohydrate side-chains (ie N-glycans) of serum glycoproteins are altered in patients with liver cirrhosis. There are increased degrees of fucosylation of serum proteins (including haptoglobin, alpha1-acid glycoprotein, and cholinesterase) in liver cirrhosis.[10] Hyposialylated variants of haptoglobin, alpha1-antitrypsin and transferrin have been detected in patients with alcoholic cirrhosis.[11]

Glycomics—the study of the global glycan profile—is a relatively new post-genome research area. When N-glycans are released from serum glycoproteins, specific types of N-glycans have been associated with cirrhosis. The unique patterns of these N-glycans have enabled the identification of cirrhosis in patients with chronic liver disease with about 80% accuracy.[12] Liver cirrhosis is the severe, end-stage of liver fibrosis, so it is possible that the aberrant N-glycans appear earlier as liver fibrosis develops, but at lower levels. Thus, the quantitative profiling of N-glycans isolated from all serum glycoprotein may enable early diagnosis and staging of liver fibrosis.

Aims and objectives

1. Establish a high-throughput assay for quantitative profiling of N-glycans attached to serum glycoproteins.

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2. Identify serum N-glycans or serum N-glycan patterns as potential biomarkers for the diagnosis of liver cirrhosis.

Methods

Quantitative profiling of serum N-glycans

N-glycans on whole serum glycoproteins were released using enzymatic digestion, cleaned by hydrophilic chromatography, and profiled with a Ciphergen ProteinChip Reader (Ciphergen Biosystems Inc, Fremont [CA], US) in linear MALDI-TOF MS mode with the use of a gold chip array and 'super-DHB' matrix (2,5-dihydroxy benzoic acid and 2-hydroxy-5-methoxybenzoic acid) supplemented with NaCl. The glycan peaks with signal-to-noise ratios of <3 among the mass spectra were quantified using the Biomarker Wizard software (Ciphergen Biosystems Inc). The peak intensities were normalised with the total ion current then with the total peak intensity. Serum samples from 40 CHB patients with, and from 40 CHB patients without, cirrhosis were subjected to the serum N-glycan profiling.

Bioinformatic analysis

To identify the glycans specifically associated with disease, two criteria were used: (1) the normalised peak intensities needed to be significantly higher/lower in patients with typical fibrosis/cirrhosis than in individuals with minimal fibrosis; and (2) the normalised peak intensities needed to correlate with the degree of fibrosis. A significance analysis of microarray algorithm[13] was used to identify the glycans that were significantly higher/lower in patients with fibrosis/cirrhosis by comparing the glycomic profiles of the patients with minimal fibrosis with those for patients with typical fibrosis/cirrhosis at a median false discovery rate of 2.5%. A forward stepwise linear regression analysis was performed to select the variables with independent prediction values for constructing a diagnostic model to calculate the Fibro-Glyco index.

Results

High-throughput assay for quantitative profiling of N-glycans attached to serum glycoproteins

A high-throughput assay was established, and its linearity and reproducibility were evaluated. N-glycans on whole serum glycoproteins were released by enzymatic digestion, cleaned by hydrophilic chromatography, and profiled with a Ciphergen ProteinChip Reader in linear MALDI-TOF MS mode with the use of a gold chip array and 'super-DHB' matrix supplemented with NaCl. By examining a mixture of four standard glycans, the intra-assay and inter-assay coefficient of variations for our assay were found to be <8% and <17%, respectively. The normalised intensities of the peaks were directly proportional to the quantity of the standard glycans with correlation coefficients of >0.96.

Identification of serum N-glycan features as biomarkers for diagnosis of liver cirrhosis

We analysed the serum N-glycan from 46 patients (29 CHB patients). For the serum samples, 63 common features were identified. A bioinformatic analysis showed that the normalised intensities of 21 different glycans correlated with fibrosis stages. Individually, a glycan of m/z 1829 ($P<0.0005$) had a sensitivity of 82% and a specificity of 84% for detecting liver fibrosis, whereas a glycan of m/z 1444 ($P<0.0005$) had a sensitivity of 76.5% and a specificity of 69% for detecting liver cirrhosis. The structures of 9 out of the 21 different glycans were predicted by searching their m/z values against a glycan mass database. Glycan species containing a proximal fucose and a bisecting N-acetyl glucosamine at the branching mannose were increased in patients with liver fibrosis and cirrhosis.

Linear regression model for detecting liver fibrosis and liver cirrhosis

Four peaks at m/z 1341.5, m/z 1829.7, m/z 1933.3 and m/z 2130.3 (all $P<0.005$) were equally effective for detecting liver fibrosis. These peaks were included (without any serological markers) in the diagnostic model. Leave-one-out cross-validation showed that the diagnostic model could identify significant fibrosis (Ishak score of ≥3) and cirrhosis (Ishak score of ≥5) with 85% accuracy.

Discussion

As only 46 patients were successfully examined in this study, we are undertaking a similar study with a much larger sample size to confirm the clinical value of the present diagnostic model. Further studies are also needed to determine whether the same serum N-glycan-based model can be used to detect liver fibrosis/cirrhosis with other underlying causes, such as CHC infection and chronic alcohol abuse.

As the m/z values only enable prediction of the structures of the diagnostic glycans, further experiments are needed to confirm the predicted structure, such as tandem mass spectrometry analysis and glycosidase sequencing.

Conclusions

A high-throughput assay was developed for the quantitative profiling of N-glycans from whole serum proteins using a system originally designed for serum proteomic profiling. This novel assay identified a panel of N-glycans as potential biomarkers for the diagnosis of liver fibrosis and liver cirrhosis. After validating the clinical values of the potential diagnostic N-glycans with a larger set of patient samples, N-glycans profiling may be used as first-line detection of liver fibrosis and liver cirrhosis, followed by biopsy for confirmation. This could alleviate the necessity of performing invasive liver biopsies on those who are unlikely to have liver fibrosis and/or liver cirrhosis.

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Prevalence of hepatitis C infection in injection drug users in Hong Kong

Key Messages

1. Hepatitis C virus (HCV) infection is highly prevalent in Hong Kong injection drug users (IDUs).
2. Infection with HCV is associated with a long history of injections, older age, and recent practice of high-risk behaviours.
3. There is a discrepancy between the low HIV rate and high HCV rate in Hong Kong IDUs, despite the common route of transmission through needle sharing.

Introduction

Injection drug use is the most common route of hepatitis C virus (HCV) transmission. In the United States, it accounted for 60% of HCV transmission.[1] High prevalence rates have been reported: 62% in Ireland,[2] 80 to 90% in Thailand,[3,4] such that HCV infection is often indicative of risk-taking injection behaviours.

The HCV situation among injection drug users (IDUs) in Hong Kong is unknown. Studies on left-over laboratory samples showed HCV antibody prevalence of 46 to 74%, with a decreasing trend over time.[5] However, the tested subjects had not been characterised in terms of risk behaviours. Whether the observed decrease in HCV rate was genuine remains speculative.

The wide network of methadone clinics has contributed to a low level of risk behaviour and thus a low HIV prevalence (less than 1%) in the IDU population.[6] In southern China, the HIV prevalence in IDUs ranged from 18 to 56% in the provinces of Guangdong and Guangxi.[7]

Aims and objectives

To establish the prevalence of HCV infection among Hong Kong IDUs and identify any associated demographic or behavioural risk factors.

Methods

Between 20 February and 27 March 2006, a survey was conducted on drug users (recruited near major methadone clinics) in clinics where blood taking and counselling could be organised, with the assistance of volunteers who were ex-heroin users. Inclusion criteria were persons who (1) had a history of injection, (2) spoke Chinese or English, (3) agreed to participate, and (4) had blood taken for HCV antibody test.

The HCV antibody test was performed by the Public Health Laboratory Centre. Specimens negative for the first enzyme immunoassay (EIA) [Murex, Dartford, UK] were considered negative, whereas those tested positive were confirmed by a different EIA kit (Ortho Diagnostic Systems, Raritan [NJ], US). A third EIA (Bioelisa, Biokit; Barcelona, Spain) was performed if the first two tests showed incongruent results. Written consent was obtained from all participating drug users. Ethical approval was obtained from the Chinese University of Hong Kong and the Department of Health. An incentive in the form of a HK$30 meal coupon was offered to each participant.

Results

Of 598 IDUs participated, 567 were included after excluding duplicates and inconsistent results. Most subjects were male (84%) and Chinese (98%). The median age was 49 years. All had a long history of drug abuse, with a median duration of 17 years.

Although most subjects received methadone treatment, three quarters admitted having injected drugs in the preceding 3 months (recent injectors). About 80%

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of the recent injectors reported using new needles for most (>50%) or all injections. About two thirds of the subjects admitted ever sharing injecting equipment, of which 15% admitted to recent sharing (in past 3 months), and 85% tested positive for HCV antibody.

Univariate analyses showed that HCV prevalence was higher in IDUs who were male, older, a longer duration of injection drug use, ever shared needles, and concurrent use of midazolam/triazolam/rohypnol. Multivariate analysis identified duration of injection, recent injection, ever sharing and use of midazolam/triazolam/rohypnol as independent factors associated with HCV seropositivity.

Discussion

Prevalence of HCV antibody was higher in IDUs than reported previously. These results may indicate that (1) all subjects had a definitive history of injection; and (2) sampling bias occurred owing to higher representation of experienced drug users. Follow-up studies are needed to establish infection rates and genotype distributions, which carry clinical and public health implications. In view of a low HIV rate in heroin users, it appears that risk behaviours tend to accumulate over the years, leading to increased HCV infection prevalence. The low HIV prevalence could be associated with a relative decline in high-risk behaviours after the 1980s, though the exact reasons need further exploration.

Conclusions

The HCV antibody prevalence in IDUs was 85%; many of them received methadone treatment. Positive HCV antibody is associated with a long history of injection, though needle-sharing practice is uncommon. Owing to the high prevalence of HCV infection in local drug users, follow-up studies (including HCV RNA tests and genotype analysis) should be useful to determine the clinical and public health implications. Treatment strategy should take into consideration of the high prevalence of HCV infection in local drug users. The discrepancy between the prevalence of HCV (85% in our study) and HIV (less than 1% from surveillance) suggests that behavioural factors alone may not account for the transmission risk in the context of public health.

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AUTHOR INDEX

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