SUPPLEMENT 8

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# HONG KONG MEDICAL JOURNAL
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## Health and Medical Research Fund
## Health and Health Services Research Fund

### Research Dissemination Reports

## 醫療衞生研究基金
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### 研究成果報告

**Mental health**
**精神健康**

**Cancer**
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**Diabetes**
**糖尿病**

**Stroke**
**中風**

**Children's health**
**兒童健康**

Supplement 8

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SUPPLEMENT 8

# MEDICAL JOURNAL 香港醫學雜誌



## Health and Medical Research Fund

## Health and Health Services Research Fund

## Research Dissemination Reports

## DIABETES



## STROKE



## CHILDREN'S HEALTH

# Editorial

Dissemination reports are concise informative reports of health-related research supported by the Health and Medical Research Fund (and its predecessor funds) administered by the Food and Health Bureau. In this edition, we present 11 dissemination reports of projects related to mental health, cancer, neurology, diabetes, stroke, and children's health. In particular, three projects are highlighted due to their potentially significant findings, impact on healthcare delivery and practice, and/or contribution to health policy formulation in Hong Kong.

Patients with unexplained neurological syndromes of the central nervous system may carry autoantibodies in their blood. Patients with such autoimmune diseases usually present with cognitive symptoms and seizure, but unexplained psychiatric presentation mimicking schizophrenia and other psychosis is not uncommon. Chong et al[1] determined the prevalence of anti-N-methyl-D-aspartate antibodies in Chinese patients with first-episode psychosis. They found that antibody prevalence was low at 1.5%. Antibody-mediated psychosis may represent a new form of mental illness requiring specific treatment. If patients with schizophrenia caused by antibodies can be treated early, there is potential to significantly improve patient outcomes and costs associated with the disorder.

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death in China and the fifth most frequent malignancy worldwide. One notable finding from cancer genome sequencing studies is the repeated discovery of somatic driver mutations in genes that encode chromatin remodelling factors, which regulate the epigenome. This suggests that changes in chromatin remodelling, which leads to epigenome disruption, is a hallmark of HCC. Cheng et al[2] investigated the role of silencing of tumour suppressor genes by trimethylation of histone H3 lysine 27 (H3K27me3) in the development of HCC following hepatitis B virus infection. Two proteins called YY1 and EZH2 were identified as crucial mediators of H3K27me3 modification. Targeting H3K27me3 epigenome for HCC prevention might benefit the large numbers of chronically HBV-infected patients.

Sporadic Parkinson disease (PD) involves a complex interplay between genetic susceptibility, environmental toxicity, and ageing. Ho et al[3] developed an experimental mouse model that carried a specific mutation at the same genetic location in parallel with humans and administered twice weekly oral doses of a naturally occurring pesticide (rotenone) over half the lifespan of the mouse to mimic chronic exposure to environmental toxicity. They found that brain abnormalities and locomotor deficits in the new model were more faithful of the human PD than other existing models. These mice may be used to test novel therapeutics for PD.

We hope you will enjoy this selection of research dissemination reports. Electronic copies of these dissemination reports and the corresponding full reports can be downloaded individually from the Research Fund Secretariat website (https://rfs2.fhb.gov.hk/). Researchers interested in the funds administered by the Food and Health Bureau also may visit the website for detailed information about application procedures.

Supplement co-editors

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1. Chong CSY, Lo WTL, Mak CM, Chen SPL, Lau KK, Sheng B. Prevalence of neuronal membrane target antibodies in first-episode psychosis: abridged secondary publication. Hong Kong Med J 2020;26(Suppl 9):S4-6.
2. Cheng ASL, Wang HT, Tsang DPF, Lee YY, Kang W, To KF. Targeting H3K27 trimethylation epigenome for liver cancer prevention: abridged secondary publication. Hong Kong Med J 2020;26(Suppl 9):S10-5.
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# Prevalence of neuronal membrane target antibodies in first-episode psychosis: abridged secondary publication

**CSY Chong *, WTL Lo, CM Mak, SPL Chen, KK Lau, B Sheng**

**KEY MESSAGES**

1. Psychosis related to anti-N-methyl-D-aspartate (NMDA) antibodies is uncommon in Hong Kong, with a prevalence of 1.5%.
2. It is clinically difficult to differentiate first-episode psychotic patients with or without anti-NMDA antibodies.
3. A high index of suspicion of psychosis related to anti-NMDA antibodies is needed for cases with unexplained abnormal electroencephalography findings.
4. Future studies may include only patients presenting as schizophrenia, owing to a potentially higher yield.

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## Introduction

Patients with unexplained neurological syndromes of the central nervous system carry autoantibodies in their blood. These conditions can occur in patients of all ages. Two such autoantibodies target voltage-gated potassium channel (VGKC)–complex proteins and N-methyl-D-aspartate (NMDA) receptors. Patients with such autoimmune diseases usually present with cognitive symptoms and seizure, but unexplained psychiatric presentation mimicking schizophrenia and other psychosis is not uncommon.[1] Common psychiatric symptoms include agitation, paranoia, psychosis, and violent behaviours. Cognitive impairments, memory deficits, speech problems, and motor symptoms can also occur. Some patients may progress to widespread encephalopathy with movement disorders, autonomic disturbance, and hypothalamic dysfunction. A pilot study in the United Kingdom explored the prevalence of the anti-VGKC and anti-NMDAR antibodies in psychotic patients who would otherwise be diagnosed with schizophrenia.[2] Of 47 patients who fulfilled DSM-IV criteria of schizophrenia, two carried anti-NMDAR antibodies, and one carried anti-VGKC antibodies, with a proportion being 6.38% (95% confidence interval=1.9%–18.9%).[2]

In addition, there is a time-sensitive response to immunotherapy in antibody-mediated cases of encephalitis.[3] If antibodies are identified and removed early, treatment response is excellent. If the disorder is associated with ovarian or testicular teratoma, the treatment response with removal of the teratoma is even better. If patients with schizophrenia caused by antibodies can be treated early, there is potential to significantly improve patient outcomes and costs associated with the disorder.

Antibody-mediated psychosis may represent a new form of mental illness requiring specific treatment. It is pertinent to determine its prevalence in patients initially diagnosed with psychosis, in whom the treatment and prognosis is completely different. We can inform health care planners of the resource implications. Associated features are highlighted to alert physicians of the possibility of autoimmune psychosis.

## Methods

The study was approved by the Research Ethics Committee of Kowloon West Cluster (Ref: KW/EX-14-054(73-09)). Consecutive subjects were recruited from the Early Intervention Team in Kwai Chung Hospital. Inclusion criteria were age of 15 to 64 years, ethnically Chinese, first-episode psychosis, with a Positive and Negative Syndrome Scale score of ≥4 on any of the positive symptoms 1, 3, 5, 6 or general symptoms 9, duration of untreated psychosis of <3 years, and <6 weeks of continuous antipsychotic medications. Subjects were excluded if they had any other neurological disorders, primary drug-induced psychosis, or failed to provide informed consent.

Basic demographic information, history of medication use, family history of autoimmune and psychotic illness, and the duration of untreated psychosis were assessed. Diagnosis was documented according to the DSM-IV-TR criteria. Blood tests including complete blood count, liver and renal function test, antinuclear antibody, and C-reactive protein were performed.

Primary outcome measurements were antibody tests for anti-NMDAR and anti-VGKC at baseline and 6 months. Secondary outcome measurements were the Positive and Negative Symptoms Scale, Catatonia Rating Scale, Addenbrookes Cognitive Examination-III, and the Social and Occupational Functioning Assessment Scale at baseline and 6 months.

## Results

A total of 341 subjects were recruited; 267 (78.3%) of them completed the second assessment at 6 months. Their mean age was 36.0 (standard deviation [SD], 13.1) years. About 64.4% of subjects were female; 50% of subjects were never married; and 51.3% of subjects were unemployed. They had a mean of 10.9 (SD, 5.1) years of education. The most common diagnosis was schizophrenia (n=136, 39.9%), followed by psychotic depression (n=79, 23.2%) and acute and transient psychosis (n=44, 14.4%). Most subjects never used illicit substances (92.7%), alcohol (89.6%), or cigarettes (82.9%). Six of them reported a history of other autoimmune disorders, namely autoimmune thyroiditis. In 52.2% of subjects, antipsychotic medications were prescribed for a mean duration of 2.3 (SD, 1.4) weeks. At baseline, the mean scores for positive, negative, and general subscales of the Positive and Negative Symptoms Scale were 17.4 (SD, 5.9), 11.3 (SD, 5.6), and 33.2 (SD, 9.1), respectively. The mean scores for Social and Occupational Functioning Assessment, Addenbrookes Cognitive Examination-III, and Catatonia Rating Scale were 47.5 (SD, 10.2), 78.5 (SD, 17.5), and 0.4 (SD, 1.1), respectively.

Only five subjects were found to be positive of anti-NMDA antibodies (Table); the overall prevalence of antineuronal antibodies in our study population was 1.5%, whereas the prevalence of antineuronal antibodies in schizophrenia and severe depressive episode with mood-congruent psychotic symptoms were 2.2% and 2.5%, respectively. No cases of anti-VGKC positivity were found.

## Discussion

The prevalence of autoimmune psychosis related to antineuronal target antibodies in our sample is much lower than that reported in overseas studies. There are several possible reasons to explain the difference. Owing to genetic and environmental differences, prevalence of autoimmune conditions varies greatly in Chinese and western populations. For example, the prevalence of rheumatoid arthritis in Hong Kong was much lower than that of European Caucasians, with a standardised morbidity ratio of 0.27 only.[4] As such, the prevalence of autoimmune psychosis may also show geographical and racial differences.

Our clinic is a tertiary referral centre for treatment of first-episode psychosis. Most patients had been assessed by other medical practitioners, with thorough organic workups. Patients with features suggestive of organic psychosis may have been referred to other specialties.

We included all cases of first-episode psychosis to better study the pattern of seropositivity. As such, we have included cases from a range of different psychiatric diagnosis. The prevalence of antineuronal antibodies has been reported to vary among different psychiatric diagnoses.[5]

In one case, positive findings were only shown at follow-up. The patient had received high-dose antipsychotic treatment for several weeks. This finding suggests that antipsychotic medication might affect the immune response and reduce antibody levels.

There are several limitations to our study. The lack of a control group limited the calculation of the relative risk of the condition. Cerebrospinal fluid samples were not collected because of the invasive procedure. Some cases with antineuronal antibodies might be missed, as around 15% of patients have antibodies only in their cerebrospinal fluid. Patients who were referred after a period of treatment elsewhere were excluded owing to possible interference of results with antipsychotic medications.

TABLE. Characteristics of the five seropositive subjects

| Sex/age of onset, y | Diagnosis | Cognitive impairment, Addenbrookes Cognitive Examination-III score | Organic workup | Electroencephalography | Antipsychotic treatment response |
|---|---|---|---|---|---|
| F/45 | Schizophrenia | Yes, 62 | Unremarkable | Generalised slow waves, frontal prominent | Good |
| F/31 | Depression with psychosis | Yes, 74 | Unremarkable | Bi-frontal slow waves | Partial |
| M/31 | Schizophrenia | Yes, 78 | Low calcium level | Spike activity over frontopolar and anterior temporal area | Partial |
| F/49 | Depression with psychosis | No | Unremarkable | - | Good |
| F/31 | Schizophrenia | - | Unremarkable | Unremarkable | Good |

## Conclusion

Electroencephalography is an important diagnostic tool for first-episode psychosis. Almost all antibody-positive cases were found to have unexplained slow activities on electroencephalography, especially in the frontal regions, while having unremarkable imaging findings.[5]

Further studies are needed to validate our findings. Future studies should include different centres in secondary and tertiary referral settings or even the emergency settings (where a number of our patients presented initially) to reduce sampling bias. Blood tests should be performed prior to the initiation of treatment, and more assessment time points may help delineate changes in antibody levels after antipsychotic treatment. A control group can help determine the prevalence of seropositivity in the general population. More proper comparison of different diagnostic groups should be performed.

## Acknowledgements

We thank Prof Belinda Lennox of the University of Oxford for her advice on the project, and Dr Leslie Jacobson from the Nuffield Department of Clinical Neuroscience, John Radcliffe Hospital for his support in the analysis of the serum samples. Our gratitude also goes to Miss Jeanie Law, Research Assistant of Kwai Chung Hospital, for her hard work in managing the project. The coordinated efforts of the staff of the Early Intervention Team of Kwai Chung Hospital, and Department of Pathology and Department of Medicine of Princess Margaret Hospital were much appreciated.

## Funding

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# Purpose-built intervention for mental health of Mainland Chinese immigrant women survivors of intimate partner violence: a randomised controlled trial (abridged secondary publication)

**A Tiwari *, DYT Fong, FKH Yuen, HYK Yuk Fung, POY Pang, JYH Wong**

## KEY MESSAGES

1. Immigration is a risk factor for intimate partner violence, and abused women are at risk of depression.
2. There is a need for evidence-based interventions to address the adverse effect of intimate partner violence on the mental health of abused Mainland Chinese immigrant women.
3. A purpose-built intervention for abused Mainland Chinese immigrant women comprising empowerment, parenting, telephone social support, and peer support can reduce depressive symptoms, lower parenting stress, improve mental health, increase perceived social support, reduce intimate partner violence, and promote safety behaviours for at least 6 months following the intervention.
4. The systematic field tracking strategies are successful in retaining study participants.

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## Introduction

Immigration is a risk factor for intimate partner violence (IPV).[1] The rate of IPV is higher among immigrant women. Men are likely to exert greater control on their immigrant partners because of the latter's dependence on them in a new country. Fear of deportation, limited knowledge of their legal rights, and lack of access to community resources may also render immigrant women more vulnerable. The lack of a social support network may further weaken a woman's ability to cope with partner violence.

This study aimed to develop a purpose-built intervention to address the needs of abused Mainland Chinese immigrant women and to assess its effect on their mental health.

## Methods

This randomised controlled study was conducted from December 2011 to March 2014 to compare a purpose-built intervention with a standard community health education programme for abused Mainland Chinese immigrant women.

The intervention lasted for 12 weeks and comprised:

(1) An empowerment component that comprised protection and enhanced choice making and problem solving based on an abuse prevention protocol.[2] Empowerment aimed to increase abused women's safety through recognition of increased danger and development of an individualised safety plan (ie protection), and to provide information about the cycle of violence, facts and options, legal protection orders, filing for criminal charges, and community resources. Women could then make decisions about their relationships, relocation, and other transitional issues (ie enhanced choice making and problem solving).

(2) A parenting component that comprised seven non-violent, positive parenting strategies (respect for the child's rights, caring and protection, supportive verbal messages, enhancing parent-child relationship, positive parental behaviours, discipline, and participation rights) based on the United Nations Children's Fund Child Friendly City Framework. Designed to enhance the knowledge, skills, and confidence of abused women in child-friendly parenting, the seven positive parenting strategies were delivered in two workshops of 2 hours each using videotapes, case studies, role play, and interactive talks.

(3) A telephone social support: three scheduled monthly telephone calls were made by our designated social worker-researchers to promote the women's health and well-being.

(4) Peer support: provided by a trained volunteer who acted as a friend to the woman and provided support through listening, validating, and facilitating in response to problems expressed by the woman. The volunteer met with the woman at least once, usually in

one of the parenting workshops, and made scheduled telephone calls, at least once a month.

For the control group, a standard community health education programme was provided. The programme comprised two group sessions lasting 2 hours each: one on the topic of osteoporosis (provided by a nurse) and one on dietary therapy based on the concepts of Chinese medicine (provided by a Chinese Medicine practitioner).

Subjects were assessed at baseline, 3 months, and 9 months, using the Beck Depression Inventory version II (BDI-II), Parenting Stress Index (PSI), Interpersonal Support Evaluation List (ISEL), SF-12 Health Survey (SF-12), Revised Conflict Tactics Scales (CTS2), and Safety Assessment Checklist (SA).

## Results

A total of 250 abused Mainland Chinese immigrant women were recruited. All but two (from the control group) completed the study. The characteristics of the intervention and control groups were comparable, except for educational level (Table 1).

TABLE 1. Characteristics of participants in the control and intervention groups*

| Characteristics | Control (n=125) | Intervention (n=125) | P value |
|---|---|---|---|
| Age (years) | 36.77±5.38 | 37.64±6.19 | 0.24 |
| Duration of living in Hong Kong (years) | 4.29±1.71 | 4.01±1.78 | 0.21 |
| No. of children | 1.68±0.86 | 1.70±0.70 | 0.84 |
| Education level | | | 0.04 |
| None | 0.1 | 1.6 | |
| Primary | 16.7 | 12.8 | |
| Junior Secondary | 76.0 | 71.2 | |
| Senior Secondary | 5.6 | 14.3 | |
| Tertiary | 1.6 | 0.1 | |
| Marital status | | | 0.32 |
| Married | 100 | 99.2 | |
| Cohabited | 0 | 0.8 | |
| Employment status | | | 0.79 |
| Employed | 36.8 | 38.4 | |
| Unemployed | 63.2 | 61.6 | |
| Financial hardship | | | 0.21 |
| Yes | 91.2 | 95.2 | |
| No | 8.8 | 4.8 | |
| Chronic illness | | | 0.65 |
| Yes | 1.6 | 1.7 | |
| No | 98.4 | 98.3 | |
| Receipt of comprehensive social security assistance | | | 0.60 |
| Yes | 34.4 | 37.6 | |
| No | 65.6 | 62.4 | |

* Data are presented as mean±SD or %

The linear mixed effect model was used to adjust for the baseline value and education level (Table 2). The adjusted mean BDI-II score was lower in the intervention than control group by 6.31 (95% CI=4.50-8.11, P<0.001) at 3 months and 8.77 (95% CI=6.96-10.59, P<0.001) at 9 months. This suggested that the intervention group had greater reduction in depressive symptoms at 9 months than at 3 months, compared with the control group (interaction, P=0.04)

The adjusted mean PSI score was lower in the intervention than control group by 11.25 (95% CI=8.53-13.97, P<0.001) at 3 months and 16.4 (95% CI=12.59-20.82, P<0.001) at 9 months. This suggested that the intervention group had greater reduction in parenting stress at 9 months than at 3 months, compared with the control group (interaction, P=0.020).

The adjusted mean ISEL score was higher in the intervention than control group by 3.65 (95% CI=2.82-4.49, P<0.001) at 3 months and 6.95 (95% CI=5.58-8.31, P<0.001) at 9 months. This suggested that the intervention group had greater improvement in perceived social support at 9 months than at 3 months, compared with the control group (interaction, P<0.001).

There was no significant difference between groups in the adjusted mean physical component score at 3 months (P=0.746) or 9 months (P=0.273), nor was there a significant interaction with time (P=0.469). In contrast, the adjusted mean mental component score was higher in the intervention than control group by 3.51 (95% CI=2.02-5.00, P<0.001) at 3 months and 6.04 (95% CI=4.06-8.02, P<0.001) at 9 months. The intervention group had better mental health at 9 months than at 3 months, compared with the control group (interaction, P=0.016).

Although there was no significant difference in adjusted mean CTS2 score between the two groups at 3 months (p=0.693), the score in the intervention group was 5.52 (95% CI=1.13-9.90, P=0.014) lower at 9 months. The intervention group had greater improvement in conflict tactics at 9 months than at 3 months, compared with the control group (interaction, P=0.01).

The adjusted mean SA score was higher in the intervention than control group by 3.69 (95% CI=3.09-4.28, P<0.001) at 3 months and 2.88 (95% CI=2.25-3.50, P<0.001) at 9 months.

## Discussion

The intervention group reported significantly fewer depressive symptoms on completion of the intervention and also 6 months post-intervention. The purpose-built intervention effectively lowered parenting stress, increased perceived social support, enhanced mental health, reduced intimate partner violence, and promoted safety behaviours among abused Mainland Chinese immigrant women in

TABLE 2. Linear mixed effect model to adjust the baseline value and education level of the intervention and control groups

| Instrument | At 3 months | | At 9 months | | P value (interaction between time-point and groups)* |
|---|---|---|---|---|---|
| | Adjusted mean difference (95% CI) | P value | Adjusted mean difference (95% CI) | P value | |
| Beck Depression Inventory version II | 6.31 (4.50-8.11) | <0.001 | 8.77 (6.96-10.59) | <0.001 | 0.04 |
| Parenting Stress Index | 11.25 (8.53-13.97) | <0.001 | 16.4 (12.59-20.82) | <0.001 | 0.02 |
| Interpersonal Support Evaluation List | 3.65 (2.82-4.49) | <0.001 | 6.95 (5.58-8.31) | <0.001 | <0.001 |
| SF-12 Health Survey | | | | | |
| Physical component score | - | 0.746 | - | 0.273 | - |
| Mental component score | 3.51 (2.02-5.00) | <0.001 | 6.04 (4.06-8.02) | <0.001 | 0.016 |
| Revised Conflict Tactics Scale | - | 0.693 | 5.52 (1.13-9.90) | 0.014 | 0.010 |
| Safety Assessment Checklist | 3.69 (3.09-4.28) | <0.001 | 2.88 (2.25-3.50) | <0.001 | - |

* The adjusted mean scores varied by group and time-point suggesting that improvement was greater at 9 months than at 3 months in the intervention group compared with the control group

Hong Kong.

A previous intervention programme for abused Chinese women comprised empowerment and social support failed to achieve clinically meaningful improvement in depressive symptoms (ie BDI-II score <5).[3] In the present study, we extended empowerment and social support by adding two extra components (parenting and peer support), and focused on abused Mainland Chinese immigrant women. The reduction of the adjusted mean BDI-II score by about 6 points at 3 months and 8 points at 6 months was clinically meaningful.

The systematic field tracking strategies could achieve a high retention rate (99.2%) in the present study, as in our previous study[3] and a study conducted in the USA.[4] We also implemented protocols to ensure participants' safety and data quality in accordance with the World Health Organization's recommendations on 'putting women first' through ethical and safe research on violence against women.[5] We anticipated, planned, and instituted safety protocols in relation to situations in which a participant and/or researcher may encounter retaliation by an abusive partner. This was particularly pertinent when contacting the woman by cell phone. Training and monitoring for researchers during the follow-up to ensure safe methods and times for contacting the women was important. Because of the risks associated with each study-related interaction, we kept such contact to the necessary minimum while using an agreed-upon script and pre-established code words. The protocols can be used to provide guidance for training and supporting research staff in future intervention studies.

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# Targeting H3K27 trimethylation epigenome for liver cancer prevention: abridged secondary publication

**ASL Cheng *, HT Wang, DPF Tsang, YY Lee, W Kang, KF To**

**KEY MESSAGES**

1. In hepatitis B virus (HBV) endemic areas such as Mainland China and Hong Kong, hepatocellular carcinoma (HCC) is a common manifestation of chronic HBV carriers. Cancer genome sequencing studies have demonstrated that epigenome disruption is a major hallmark of HCC.
2. The HBV X protein (HBx) has been shown to up-regulate the polycomb protein enhancer of zeste homolog 2 (EZH2), which catalyses tumour suppressor gene silencing via histone H3 lysine 27 trimethylation (H3K27me3). The identification of genomic repertoire of H3K27me3 targets and the polycomb recruiters for H3K27me3 deposition provide insights into molecular carcinogenesis and development of novel therapeutic strategies.
3. Using an integrated high-resolution genome-wide approach, we have characterised the HBx-deregulated H3K27me3 epigenome in HBx-transgenic HCC model. Our integrative study demonstrates that Ying yang 1 overexpression contributes to EZH2 recruitment for H3K27me3-mediated repression of tumour-suppressive protein–coding and microRNA genes, thereby enhancing nuclear factor-kappa B signalling in hepatocarcinogenesis.
4. Despite the survival improvement in HCC patients receiving multi-kinase-targeted inhibitor, the outcomes are still far from satisfactory. Given the availability of potent EZH2 inhibitors and their significant effects in reactivating tumour suppressor genes in HCC cells, further testing is warranted to determine if it is an effective chemopreventive strategy in patients with HBV-associated precancerous lesions. Targeting H3K27me3 epigenome for HCC prevention might benefit large populations of chronically HBV-infected patients.

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## Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death in China and the fifth most frequent malignancy worldwide. Most HCC cases are associated with cirrhosis secondary to chronic hepatitis B virus (HBV) infection. Around 12% of the population of China are estimated to be HBV carriers. Although targeted therapy using tyrosine kinase inhibitor has shown clinical efficacy, no specific oncogene addictions are yet known for HCC.

One remarkable finding of cancer genome sequencing is the repeated discovery of somatic driver mutations in genes that encode chromatin-remodelling factors, which regulate the epigenome (ie the totality of chemical modifications to the genome that do not involve a change in the nucleotide sequence). As much as 50% of HCCs are estimated to harbour mutations in different chromatin regulators. This suggests that aberration in chromatin remodelling, which leads to epigenome disruption, is a hallmark of HCC.[1] In addition to somatic mutations, malfunction of chromatin regulators can be caused by transcriptional deregulation in cancer. Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of the polycomb-repressive complex 2 that represses gene transcription through histone H3 lysine 27 trimethylation (H3K27me3).[2] *EZH2* gene mutations are discovered in haematological cancers, and aberrant mis-expression of EZH2 is noted in a variety of solid tumours including HCC.[3] The HBV X protein (HBx), a promiscuous *trans*-activator involved in hepatocellular neoplastic transformation, can up-regulate EZH2 gene expression.[3,4] Although EZH2 causes H3K27me3-mediated silencing of Wnt antagonists to promote β-catenin-dependent hepatocarcinogenesis,[2,3] the complete genomic repertoire of H3K27me3 targets and their deregulated signalling pathways in HCC remain largely unknown. To determine the epigenetic connection between chronic HBV infection and hepatocarcinogenesis, we used an HBx-transgenic (TG) HCC model

to characterise the H3K27me3 epigenome using an integrated high-resolution genome-wide and bioinformatics approach. Our findings reveal new epigenetically-silenced microRNAs (miRNAs) that contribute to key oncogenic signalling activation in HCC development.

## Methods

Liver tissues from both HBx homozygous TG and C57BL/6 wild-type male mice were collected for immunoprecipitation of H3K27me3-bound DNA followed by high-throughput DNA sequencing (ChIP-seq) and RNA-seq.[5] The animal study was approved by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong.

Human HCC cell lines were transfected with small-interfering RNAs using HiPerfect (Qiagen).[3,4] For measuring the activities of cancer-related transcription factors, cells were transfected with the cancer 10-pathway reporter constructs (Qiagen) followed by dual luciferase reporter assays (Promega).[5] For validation of miRNA targets, wild-type or mutated construct was cloned into the vector pGL3-basic (Promega) followed by transfection and dual luciferase reporter assays.

Quantitative RT-PCR, western blot, and immunohistochemistry in human HCC tissue microarray were performed as previously described.[3-5]

Patients who underwent hepatectomy for HCC at the Prince of Wales Hospital, Hong Kong were included. All patients gave written informed consent on the use of clinical specimens for research purposes. This study was approved by the Joint Chinese University of Hong Kong – New Territories East Clinical Research Ethics Committee.

Data were expressed as mean ± standard deviation with triplicate experiments. Independent Student's *t*-tests were used for in vitro experiments. Differences in gene expression levels in clinical samples was analysed using paired *t*-tests. Kaplan Meier survival analysis was used to determine the disease-free survival rate; the difference was compared by log-rank Mantel-Cox test.

## Results

We used ChIP-seq and RNA-seq to investigate the genome-wide H3K27me3-bound promoters and the concurrently down-regulated genes in normal and tumour-bearing liver tissues from wild-type and HBx-TG mice (Fig 1a). Western blot analysis showed that the normal livers from wild-type mice contained low levels of EZH2 and H3K27me3. By contrast, the liver tissues from ageing HBx-TG mice exhibited increasing expressions of these proteins and peaked in the tumour tissues of 18-month-old TG mice (Fig 1b). By integrating the ChIP-seq and RNA-seq results, we revealed 1359 and 1027 H3K27me3-occupied and repressed genes in tumour (TG-18m-T) versus normal liver (WT-3m-NL) and adjacent non-tumour (TG-18m-NT), respectively (Fig 1c). Among the 611 targets common in both gene lists, at least 20 have been reported to be TSGs (Fig 1d). For example, potential H3K27me3-mediated silencing of *glycine N-methyltransferase (gnmt)* was a prominent tumour suppressor in murine and human HCCs (Fig 1e).

Quantitative RT-PCR demonstrated that 16 potential TSGs were significantly down-regulated in murine tumours compared with control tissues (Fig 1f). Pharmacological inhibition of EZH2 and H3K27me3 in four human HCC cell lines further demonstrated that nine of the 16 genes were indeed epigenetically regulated (data not shown). To investigate the clinical relevance of our findings, we examined their gene expressions in 12 pairs of human HBV-associated HCC specimens, and six genes were significantly down-regulated in tumours compared with adjacent non-tumour counterparts (Fig 1g). These data illustrate that EZH2 epigenetically silences TSGs via H3K27me3 during HCC development.

To elucidate the transcription factors that mediate polycomb targeting in HCC, we performed motif analysis of the tumour-specific H3K27me3-bound regions and identified a highly significant centrally-enriched motif (P<5E-6) for YY1. Western blot analysis of the wild-type and HBx-TG tissues showed concordant up-regulation of YY1, EZH2, and H3K27me3 in HCC tissues, thus supporting the involvement of YY1 in H3K27me3 modification (Fig 1b). Down-regulation of YY1 remarkably reduced the global levels of H3K27me3 in HCC cell lines (Fig 2a). Using 50 pairs of human HBV-associated HCCs, quantitative RT-PCR demonstrated that YY1 and EZH2 overexpression was detected in 68% and 84% of HCCs (Fig 2b) and positively correlated (P<0.0001, Fig 2c). Consistently, both protein expressions were significantly up-regulated in HCCs as shown by immunohistochemistry using a tissue microarray containing 194 cases (P=9E-5 and P=2.4E-44, respectively, Fig 2d). Kaplan–Meier analysis showed that advanced-stage (II-III) HCC patients with concomitant YY1/EZH2 up-regulation were significantly associated with shorter disease-free survival (hazard ratio=1.875, 95% confidence interval=1.008-3.488, P=0.047, Fig 2e).

Given the extensive H3K27me3 modification in the HCC genome, we focused on aberrant epigenetic control of miRNAs because of their biological significance in signalling deregulation. ChIP-seq demonstrated enriched H3K27me3 occupancy in 16 miRNA loci in HBx-induced HCC tumours compared with the adjacent non-tumour tissues (Fig 3a). Notably, 15 of them have been reported

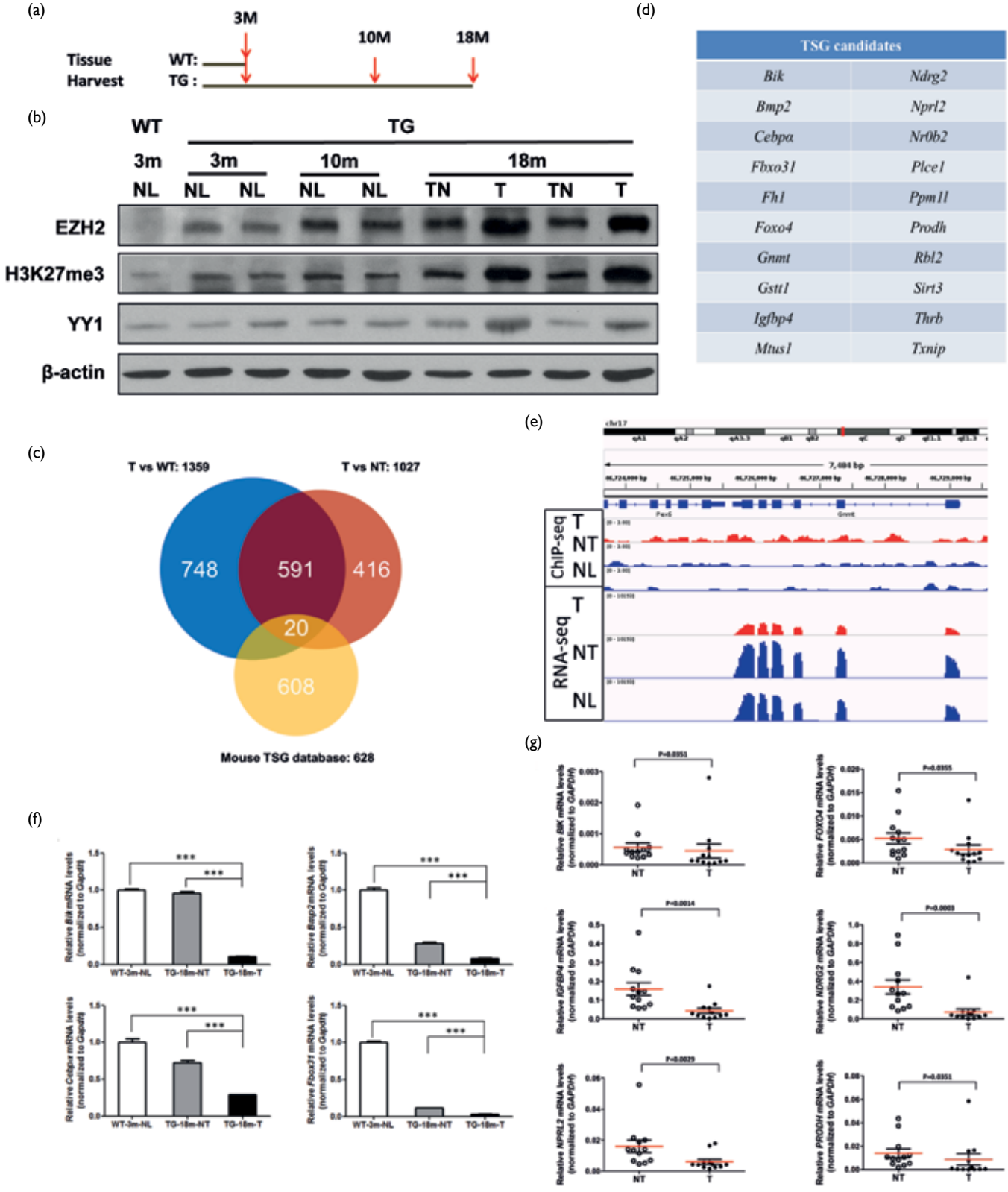


**FIG 1. Deregulation of H3K27 trimethylation and gene repression in an HBx-TG mouse model:** (a) Schematic diagram showing the collection of liver tissues from wild-type and HBx-TG mice at different time-points. (b) Western blot analysis of the normal (NL), adjacent non-tumour (NT), and tumour (T) tissues. (c) Venn diagram showing the integration of ChIP-seq, RNA-seq and mouse TSG database: 1359 H3K27me3-occupied and repressed genes in tumour (TG-18m-T) versus normal liver (WT-3m-NL); 1027 genes in tumour (TG-18m-T) versus adjacent non-tumour (TG-18m-NT); and 628 mouse TSGs from a published database. (d) 20 epigenetically-regulated TSGs in murine HBx-TG model. (e) Representative diagram showing the ChIP-seq and RNA-seq signals mapped to a candidate gene, Gnmt, in liver tissues of WT-3m-NL, TG-18m-NT and TG-18m-T mice, respectively. Quantitative RT-PCR analysis of the candidate genes in the (f) original murine tissues and (g) human HBV-associated HCCs. **P<0.01; ***P<0.001.

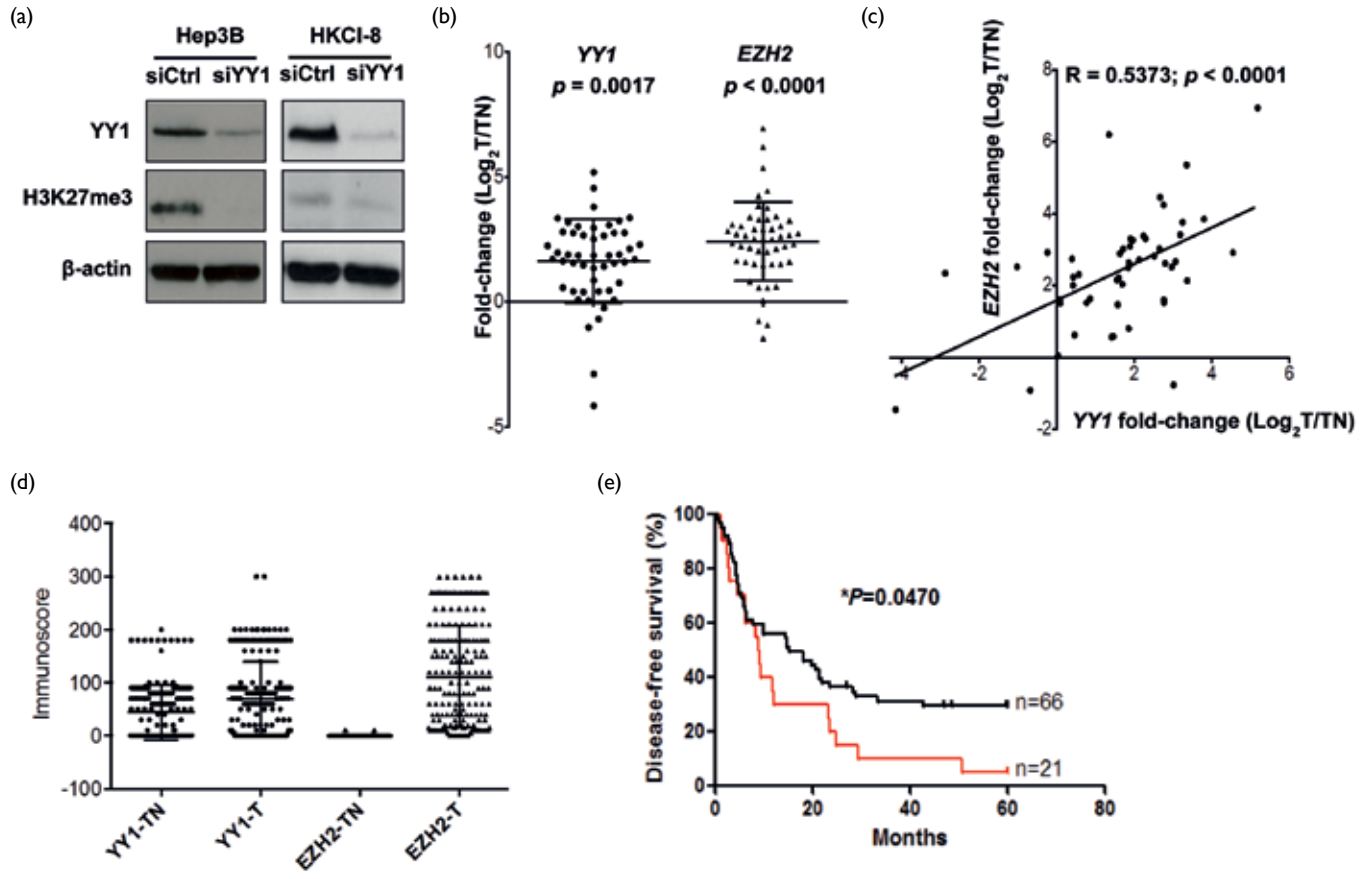


**FIG 2. Significant association between YY1 and H3K27me3 in HCC:** (a) Western blot showing that knockdown of YY1 reduced H3K27me3 levels in Hep3B and HKCI-8 HCC cells. (b) Up-regulation of *YY1* and *EZH2* mRNA in 68% and 84% of human HCCs, respectively. (c) *YY1* and *EZH2* gene expressions exhibiting a significant positive correlation. (d) Immunoreactivities of YY1 and EZH2 are significantly higher in human HCC tumours (T) than in tumour-adjacent liver tissues (TN). (e) Survival of advanced-stage HCC patients with concurrent up-regulation (high activation) of YY1 and EZH2 is significantly shorter than those with low activation.

to function as TSG in human cancers (Fig 3b). We found that knockdown of YY1 not only decreased its own occupancy but also EZH2 and H3K27me3 in the upstream regions of the *miR-9-1* and *miR-9-2* loci (P<0.05, Fig 3c), resulting in *miR-9* transcription in HCC cells (P<0.01, Fig 3d).

Using a luciferase reporter array that allows simultaneous measurement of ten transcription factor activities, we found that YY1 knockdown significantly reduced NF-κB signalling by ~50% in HCC cells (P<0.01, Figs 3e, 3f). Consistently, down-regulation of YY1 suppressed the p50 and p65 protein expressions (Fig 3e). Using TarBase 6.0 database, we found that the target genes of the 16 H3K27me3-occupied miRNAs were enriched in the regulation of the IκB kinase/NF-κB cascade (P=0.00156). We experimentally confirmed the direct repression of *NFKB1* by *miR-9* in HCC cells (Fig 3g). Concordantly, *miR-9* overexpression reduced the protein levels of p50 and p65 (Fig 3h) and NF-κB transcriptional activity (Fig 3i). Collectively, these data suggest that epigenetic silencing of miRNAs by YY1 activates NF-κB signalling in HCC cells.

## Discussion

Genome-wide mapping of polycomb binding sites in different cell types and disease states has revealed an unexpected diversity. The identification of the PRC2 recruiters for H3K27me3 deposition provides additional insights into molecular pathogenesis and development of novel therapeutic strategies. Using an HBx transgenic model, HBV-associated HCC cell lines, and clinical specimens, we provide evidence to support the notion that YY1 acts as a crucial mediator of H3K27me3 modification in HCC. Of particular clinicopathological significance, we found that concomitant up-regulation of YY1 and EZH2 is associated with shortened survival in patients with advanced HCC. Multiple tumour-suppressive protein-coding and miRNA genes are concordantly silenced by H3K27me3, the latter of which

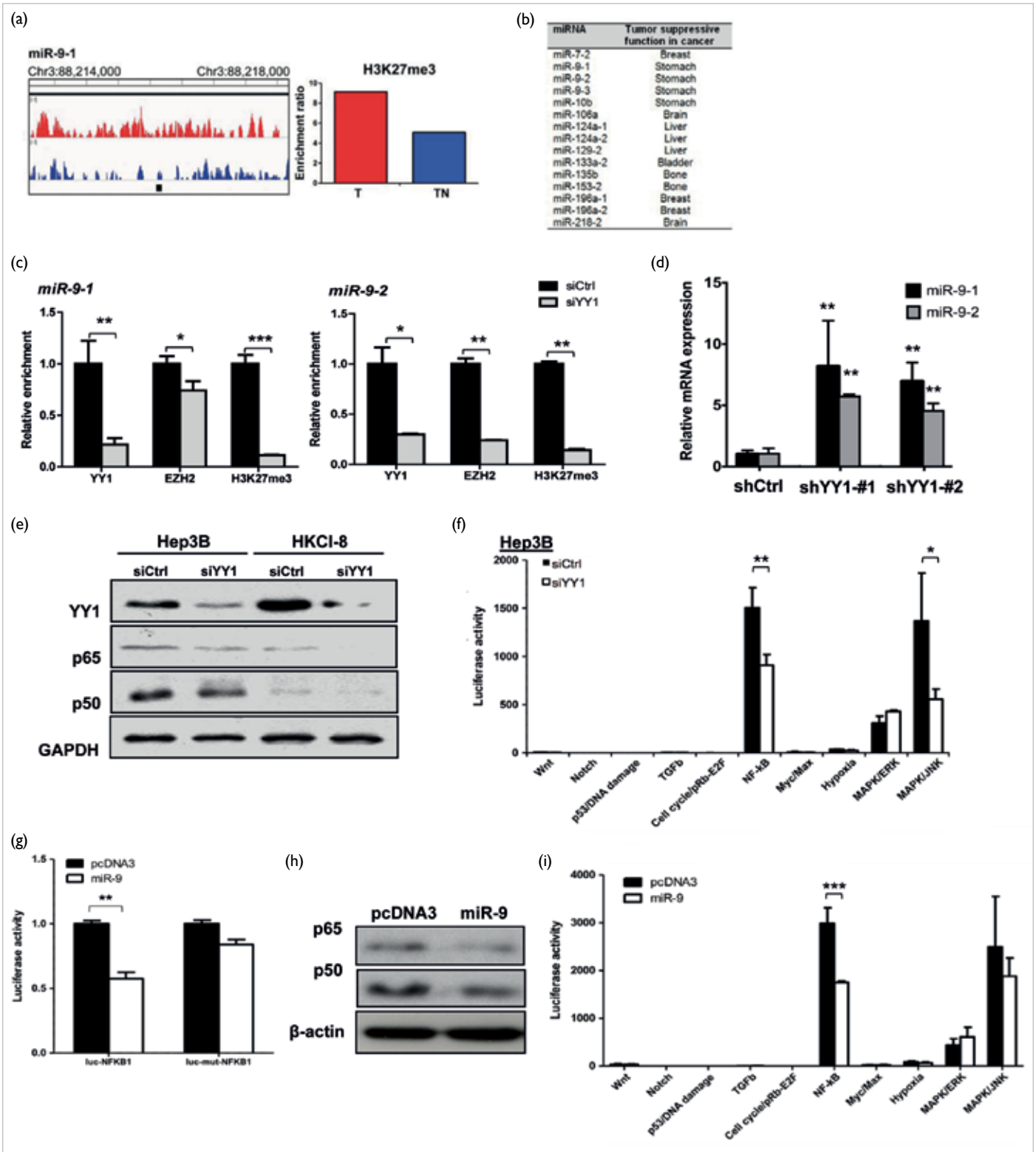


**FIG 3. Epigenetic silencing of miRNA by YY1 activates NF-κB signalling in HCC cells:** (a) H3K27me3 enrichment at miRNA loci in HCC compared with non-tumour tissues as revealed by ChIP-seq. (b) List of H3K27me3-enriched miRNAs in HBx-TG model showing tumour suppressive functions in human cancers. (c) Quantitative ChIP-PCR analysis of YY1, EZH2, and H3K27me3 occupancy in the promoter regions. (d) Quantitative RT-PCR analysis of *miR-9-1* and *miR-9-2* upon YY1 knockdown in Hep3B cells. (e) Western blot analysis of p65 and p50 upon YY1 knockdown in Hep3B and HKCI-8. (f) The activities of ten cancer-related transcription factors upon YY1 knockdown were measured by luciferase reporter assays. (g) Luciferase reporter assays confirmed the direct physical interaction between miR-9 and NFKB1 3'UTR in Hep3B. (h) Western blot analysis of p65 and p50, and (i) luciferase reporter assay in Hep3B upon transfection with miR-9 mimics. * P<0.05; ** P<0.01; *** P<0.001.

contributes to constitutive NF-κB activation in HCC cells. These findings not only unveil a novel master regulator of PRC2 recruitment in HCC but also shed mechanistic insight into the anti-neoplastic action of EZH2-targeted therapy.

Activation of NF-κB, a master regulator of inflammation and cell survival, is a frequent and early event in human HCC of both viral and non-viral aetiologies. The carcinogenesis function of NF-κB is supported by genetically-modified mouse studies. We showed that YY1 overexpression contributes to NF-κB activation in HCC, which is at least partially mediated by epigenetic deregulation of miRNAs. To our knowledge, this is the first evidence to support a chromatin regulation of NF-κB signalling via miRNAs in HCC.

This project has improved our understanding on the establishment and functional significance of H3K27me3 epigenome in HBV-associated HCC. Our integrative study demonstrates that YY1 overexpression contributes to EZH2 recruitment for H3K27me3-mediated repression of tumour-suppressive protein-coding and miRNA genes, thereby enhancing key oncogenic signalling pathways in hepatocarcinogenesis.[4,5] With the advancement of genome editing technology, liver cancer cell-specific targeting of YY1 could be a novel therapeutic option. Given the promising preliminary data from ongoing clinical trials of specific EZH2 inhibitors in different haematological and solid tumours (NCT01897571, NCT02082977, NCT02601950), the strategy of targeting H3K27me3 epigenome for HCC prevention and treatment might benefit large populations of chronically HBV-infected patients.

## Funding

This study was supported by the Health and Medical Research Fund, Food and Health Bureau, Hong Kong SAR Government (#01120216). The full report is available from the Health and Medical Research Fund website (https://rfs1.fhb.gov.hk/index.html).

## Disclosure

The results of this research have been previously published in:

1. Tsang DP, Wu WK, Kang W, et al. Yin Yang 1-mediated epigenetic silencing of tumour-suppressive microRNAs activates nuclear factor-κB in hepatocellular carcinoma. J Pathol 2016;238:651-64.

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# Ginsenoside-Rb1 as an anti-cancer therapeutic: abridged secondary publication

**AST Wong *, CKC Wong**

**KEY MESSAGES**

1. Ginsenoside Rb1 and its derivative compound K target cancer stem cells.
2. Ginsenoside Rb1 and compound K chemosensitise cancer stem cells to chemotherapeutic drugs.
3. Wnt/β-catenin signalling causes the ensuing cytotoxic effects.

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## Introduction

Recurrence of cancer despite chemotherapy is associated with high mortality. Cancer stem cells (CSCs) have been reported to possess properties of self-renewal, differentiation, and drug resistance that lead to tumorigenesis and chemoresistance.[1] They are not eliminated by conventional chemotherapy owing to their distinct molecular signatures. Thus, understanding CSCs' biology and targeting them are important.

Saponins have been shown to exhibit potent cytotoxic effects as chemotherapeutics.[2] Ginsenoside-Rb1 isolated from ginseng (0.37%-0.5%) is a notable saponin. After being taken orally, about 70% of Rb1 is metabolised to 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol (compound K) by gut microbes.[3] This metabolite can be readily absorbed into the blood and retained in the body.

## Methods

SKOV-3 and HEYA8 CSCs or primary CSCs derived from patients with ovarian carcinoma were isolated in serum-free stem cell-selective conditions and cultured as previously described.[4] To assess tumour recurrence, primary spheres were dissociated as single cells and replated for sphere formation by secondary passaging.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to analyse the rates of proliferation/survival based on the manufacturer's protocols (Sigma, St Louis [MO], US). The quantity of formazan was measured at a wavelength of 570 nm using a microplate reader (Bio-Rad, Hercules [CA], US).

Equal amounts of proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and blocked with 5% non-fat milk for 1 hour at room temperature. Membranes were then incubated with the primary antibody overnight at 4°C, followed by incubation with horseradish peroxidise-conjugated secondary antibodies for 3 hours at room temperature (Bio-Rad, Hercules [CA], US). β-actin was included as a loading control. The target proteins were detected by Amersham enhanced chemiluminescent detection reagents (GE Healthcare, Little Chalfont, UK).

All animal experiments were approved by the University of Hong Kong Institutional Animal Care and Use Committee. Cells ($10^6$ cells) were inoculated subcutaneously into the right flank of BALB/c athymic nude mice. When tumour size reached approximately 50 $mm^3$, the mice were randomly divided into nine groups and administered DMSO, Rb1, compound K, cisplatin, paclitaxel, Rb1 + cisplatin, Rb1 + paclitaxel, compound K + cisplatin, or compound K + paclitaxel by oral gavage. Tumour volume and body weight were measured twice weekly for 28 days. Blood samples were collected from the retro-orbital sinus to test enzyme levels indicative of cardiac, hepatic, and renal function. Organs were paraffin embedded and stained with haematoxylin and eosin for histologic examination.

One-way analyses of variance were used to compare different treatment groups. The Tukey test was performed to detect differences. P values of $<0.05$ were considered statistically significant.

## Results

MTT assay was used to examine the cytotoxic effects of Rb1 and its metabolite compound K on SKOV-3 and HEYA8 CSCs. Rb1 and compound K inhibited tumour sphere formation and growth of both SKOV-3 and HEYA8 CSCs in a dose-dependent manner. The lethal concentrations that led to 50% survival in SKOV-3 cells were 0.25 μM for Rb1 and 0.1 μM for compound K, whereas in HEYA8 cells, the corresponding lethal concentrations were 0.23 μM for Rb1 and 0.125 μM for compound K. In addition, 250 nM Rb1 and 125 nM compound K suppressed tumour sphere formation and growth of SKOV-3 and HEYA8 CSCs in a time-dependent manner (measured at 0, 24, and 48 hours). Moreover, Rb1 and compound K blocked the regrowth of

secondary spheres. The expression of CSC markers such as Bmil-1, Oct4, and Nanog were reduced by Rb1 or compound K, with maximal effects after 48 hours. These results suggest that Rb1 and compound K inhibit CSC self-renewal.

We further investigated the effects of Rb1 and compound K on chemotherapeutics. In SKOV-3 and HEYA8 CSCs, Rb1 or compound K sensitised CSCs to clinically relevant doses of cisplatin (50 μM) and paclitaxel (100 nM).[5] Consistently, the expression levels of Bmi-1, Oct4, and Nanog decreased. In contrast, SKOV-3 and HEYA8 CSCs were resistant to both cisplatin and paclitaxel without treatment by Rb1 or compound K.

To explore the underlying mechanism by which Rb1 and compound K regulate CSC chemosensitisation, the key role of Wnt/β-catenin signalling in CSC self-renewal and carcinogenesis was determined. Rb1 and compound K significantly inhibited β-catenin expression concomitant with a decrease in β-catenin/TCF transcriptional activity via TOPFLASH activation. There was a decrease in the expression of two β-catenin/TCF targets and pivotal drug transporters ABCG2 and P-glycoprotein. Nonetheless, expression of a stable mutant form of β-catenin (S37A) reverted the chemosensitising effect of Rb1 and compound K in CSCs. Similar results were obtained by expressing a constitutively active construct of TCF (VP16-TCF). These results suggest that Rb1 and compound K target the Wnt/β-catenin-ABCG2 and P-glycoprotein signalling pathway.

An in vivo study was performed to further assess the effects of Rb1 and compound K. Tumour growth was significantly inhibited by Rb1 or compound K compared with vehicle controls. The combined treatment of Rb1 or compound K with cisplatin or paclitaxel led to a marked decrease in tumour size. There was no noted weight loss in the animals, and there was no histological or enzymatic alteration in the heart, kidney, liver, lungs, and spleen compared with controls. These results suggest that Rb1 and compound K could be used as anti-CSC agents with no obvious adverse effects.

## Discussion

Chemoresistance is a major factor that leads to failure of anti-cancer therapies. In this study, Rb1 and compound K significantly inhibited the growth of CSCs and enhanced sensitivities to cisplatin and paclitaxel through the Wnt/β-catenin-ABCG2/P-glycoprotein signalling pathway. To our knowledge, this is the first evidence that ginseng affects CSC chemosensitisation. Combined therapy could be beneficial for refractory/recurrent tumours.

Rb1 and compound K could be promising therapeutic and chemopreventive agents. They exhibit better bioavailability than most other saponins, and they show various anti-carcinogenic activities such as growth inhibition, apoptosis, and anti-angiogenesis. They inhibit many tumour-promoting activities and seem to improve normal cellular functions.

Cisplatin and paclitaxel are major anti-cancer drugs for treatment of ovarian cancer. Nonetheless, drug resistance remains a major challenge. Rb1 and compound K could sensitise CSCs, the drug-resistant subpopulation of cancer cells, to clinical doses of cisplatin and paclitaxel chemotherapeutic treatments.

Ginseng has been considered to have low toxicity, and overdose is well tolerated. Rb1 and compound K should be further investigated as an anti-cancer therapeutics, as there was no organ toxicity *in vivo*.

## Funding

This study was supported by the Health and Medical Research Fund, Food and Health Bureau, Hong Kong SAR Government (#11121191). The full report is available from the Health and Medical Research Fund website (https://rfs1.fhb.gov.hk/index.html).

## Disclosure

The results of this research have been previously published in:

1. Wong AS, Che CM, Leung KW. Recent advances in ginseng as cancer therapeutics: a functional and mechanistic overview. Nat Prod Rep 2015;32:256-72.
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# Fast-track versus traditional perioperative care for laparoscopic colorectal surgery: a prospective randomised trial (abridged secondary publication)

**SSM Ng *, SKC Chan, MHL Ng, JFY Lee, PBS Lai**

**KEY MESSAGES**

1. Compared with traditional perioperative care, fast-track (FT) perioperative care for laparoscopic surgery for colorectal cancer resulted in faster clinical recovery, reduced morbidity, less stress response, and better-preserved cell-mediated immunity.
2. FT perioperative care was an independent predictor of shorter total postoperative hospital stay.
3. The total direct cost was significantly lower in patients who received FT perioperative care.

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## Introduction

Compared with open surgery, laparoscopic colorectal surgery is associated with better short-term clinical outcome in terms of pain, gastrointestinal recovery, and hospital stay. In a traditional perioperative care setting, the reduction in hospital stay following laparoscopic surgery is modest.[1] Laparoscopic colorectal surgery requires a long operative time and expensive disposable instruments. As such, its cost-effectiveness is a major concern for hospital administrators and policy-makers.

Fast-track (FT) perioperative care can reduce surgical stress and enhance recovery following colorectal surgery.[2] Hospital stay after open colectomy can be reduced to 2 to 3 days with the FT perioperative care. Nonetheless few studies have evaluated the impact of FT perioperative care on the clinical and immunological outcome of laparoscopic colorectal surgery.[3,4] We aimed to evaluate the clinical (hospital stay and quality of life) and immunological (systemic cytokine response and cell-mediated immune function) outcome in Hong Kong Chinese patients who underwent laparoscopic surgery for colorectal cancer and received either FT or traditional perioperative care.

## Methods

### Study participants

This study was conducted from December 2010 to March 2013. The study protocol was approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee, and registered with ClinicalTrials.gov (NCT01341366). Inclusion criteria were consecutive patients aged 18 to 80 years with American Society of Anesthesiologists grade I to III who underwent elective laparoscopic resection of colonic or upper rectal cancer. Patients were excluded if they underwent laparoscopic resection of mid or low rectal cancer, complex/combined laparoscopic procedures, or emergency surgery, had a stoma created, developed intraoperative problems that required conversion, or had known metastatic disease or a history of midline laparotomy.

### Study design

Patients were randomised to receive FT or traditional perioperative care, stratified for right-sided or left-sided colorectal resection. A sealed opaque envelope (according to the computer-generated random sequence) was used to determine the appropriate programme of care. All laparoscopic surgeries were performed by experienced colorectal surgeons. Clinical outcome was assessed by an independent research assistant daily from day 0 until the day of discharge. Discharge criteria were similar for both groups, and consisted of adequate pain control, ability to tolerate solid food, ability to mobilise independently, and acceptance of discharge by the patient. Patients were telephoned daily by a designated registered nurse until review at the outpatient clinic on day 14. Patients were seen again at the outpatient clinic at 4 and 12 weeks.

### Interventions

Details of the FT and traditional perioperative care are summarised in Table 1. The FT perioperative care was based on a consensus among our surgeons, anaesthetists, physiotherapists, dieticians, and nurses

TABLE 1. Comparison of the fast-track and traditional perioperative care

| Phase | Fast-track perioperative care | Traditional perioperative care |
|---|---|---|
| Preoperative | | |
| Colorectal clinic (surgery) | • Scheduling of surgery<br>• Information about fast-track perioperative care, discussing discharge on day 5 if possible<br>• Informed consent | • Scheduling of surgery<br>• Informed consent |
| Preoperative clinic (anaesthesia) | • Pre-assessment for risk adjustment<br>• Discussion focusing on fast-track anaesthetic and postoperative pain management<br>• Explanation of pain assessment using visual analogue scale (VAS) | • Pre-assessment for risk adjustment<br>• Explanation of intravenous patient-controlled analgesia (PCA) for postoperative pain management<br>• Explanation of pain assessment using VAS |
| Preadmission counselling and guided tour on surgical ward | • Yes | • No |
| Admission day | | |
| Bowel preparation | • Klean-Prep (Norgine Ltd., Middlesex, UK) x 4 L | • Klean-Prep (Norgine Ltd., Middlesex, UK) x 4 L |
| Diet | • Last meal 6 hours before surgery | • Last meal by midnight the day before |
| Preoperative carbohydrate-loaded drink | • Polycal Powder (Nutricia Advanced Medical Nutrition, UK) x 500 mL the evening before surgery | • No |
| Surgery day | | |
| Pre-anaesthetic medication | • No | • No |
| Anaesthetic management | • Induction with fentanyl 1 μg/kg, propofol 2 mg/kg, and rocuronium 0.6 mg/kg<br>• Anaesthesia maintained with propofol infusion 4-8 mg/kg/h and remifentanil infusion 0.05-0.2 μg/kg/min<br>• Ventilation maintained with 40% oxygen in air<br>• Forced body heating (Bair Hugger system and warmed intravenous fluids)<br>• Give tramadol 1 mg/kg intravenously upon skin incision<br>• Give parecoxib 40 mg intravenously after skin closure<br>• Prophylactic use of ondansetron 4 mg intravenously to prevent postoperative nausea and vomiting<br>• Intraoperative fluid restriction of crystalloid solution to 10 mL/kg and titrate with urinary output of >0.5 mL/kg/h<br>• Intraoperative blood loss will be replaced with colloid solution | • Induction with fentanyl 1 μg/kg, propofol 2 mg/kg, and rocuronium 0.6 mg/kg<br>• Anaesthesia maintained with sevoflurane 0.5-1.5% and oxygen 40% in nitrous oxide<br>• Forced body heating (Bair Hugger system and warmed intravenous fluids)<br>• Give morphine 0.1 mg/kg intravenously upon skin incision<br>• Use of metoclopramide for postoperative nausea and vomiting according to list anaesthetist<br>• No restriction on intraoperative fluid management |
| Surgical management | • Minimally invasive incisions<br>• Infiltration of wounds with 0.5% levobupivacaine 0.2 mL/kg<br>• Continuous wound instillation with 0.5% levobupivacaine using the ON-Q PainBuster System (I-Flow Corporation, Lake Forest, CA, USA) at 2 mL/h for 48 h<br>• Urinary catheter<br>• Use of abdominal drain | • Minimally invasive incisions<br>• No infiltration of surgical wounds with local anaesthetic drugs<br>• Urinary catheter<br>• Use of abdominal drain |
| Early postoperative management | • Give incremental doses of fentanyl 10 μg intravenously if severe pain in recovery room<br>• Add oral tramadol 50 mg plus paracetamol 1 g 3 times per day for 3 days as postoperative analgesia<br>• Consider oral or intramuscular tramadol 50 mg for rescue pain if VAS ≥4<br>• First oral drink at 2 h after surgery + intravenous infusion of crystalloid solution 1.5 L/day<br>• Sit out in chair in the evening (>2 h out of bed) | • Give incremental doses of morphine 1 mg intravenously if severe pain in recovery room<br>• Postoperative analgesia provided by intravenous PCA morphine for 3 days (add oral tramadol 50 mg plus paracetamol 1 g 3 times per day from day 2 onwards)<br>• 'Nil by mouth' + intravenous infusion of crystalloid solution 2 L/day<br>• No mobilisation scheme |
| Day 1 | • Offer soft diet<br>• 'Extra' sugarfree gum 3 times per day<br>• Stop intravenous fluid (leave cannula)<br>• Remove urinary catheter<br>• Expand mobilisation (>6 h out of bed) | • Sips of water' orally<br>• No chewing gum<br>• Intravenous fluid administration 2 L/day<br>• Mobilisation according to attending surgeon |
| Day 2 | • Offer normal diet<br>• 'Extra' sugarfree gum 3 times per day<br>• Remove intravenous cannula<br>• Remove drain<br>• Expand mobilisation (>8 h out of bed) | • Diet increases on daily basis<br>• No chewing gum<br>• Intravenous fluid administration is continued until adequate oral intake<br>• Removal of urinary catheter and abdominal drain at discretion of attending surgeon<br>• Mobilisation according to attending surgeon |
| Day 3 | • Continue as on day 2 until discharge criteria are fulfilled | • Continue as on day 2 until discharge criteria are fulfilled |

who had reviewed the relevant evidence and made appropriate adjustments to suit the local situation.[2-4]

### Determination of immunological outcome

Peripheral venous blood samples were taken preoperatively and at 2 hours, 8 hours, 24 hours, 48 hours, and 5 days after surgery for measurement of systemic cytokine and cell-mediated immune responses. Interleukin-1β (IL-1β) and interleukin-6 (IL-6) were measured in triplicate by enzyme-linked immunosorbent assay. C-reactive protein (CRP) was measured by immunoturbidimetry. Lymphocyte subsets were measured using FACSCalibur flow cytometer after immunophenotyping.

### Main outcome measures

The primary outcome measure was total postoperative hospital stay (including that of patients readmitted within 30 days of surgery). The secondary outcome measures were (1) immunological parameters, including cytokine and CRP levels, and lymphocyte subsets, (2) pain score on a visual analogue scale, (3) morbidity and mortality within 30 days, (4) readmission rate, (5) quality of life at 2 and 4 weeks measured by the SF-36 questionnaire, and (6) direct medical costs.

### Statistical analysis and sample size estimation

Data were analysed according to the intention-to-treat principle. Confounders that might affect length of hospital stay were adjusted using multiple linear regression analysis. A two-sided P value <0.05 was considered statistically significant.

The mean hospital stay for laparoscopic resection of colonic and upper rectal cancer with traditional perioperative care in patients aged <80 years was 8 (standard deviation, 6) days at our institution. Assuming that the FT perioperative care could reduce the hospital stay to 5 days, a sample size of 64 patients in each group was needed to yield a power of 80% with a significance level of 0.05.

## Results

Between December 2010 and March 2013, 157 consecutive patients were assessed for eligibility. Of these, 22 were excluded and 135 were randomised to receive FT (n=68) or traditional (n=67) perioperative care. Four patients in the FT group and three patients in the traditional group were excluded after randomisation because of conversion to laparotomy. No patients were withdrawn or dropped out. Baseline characteristics of the two groups were comparable (Table 2).

### Primary outcome measure

Compared with the traditional group, the FT group had shorter median postoperative hospital stay (4 vs 5 days, P<0.001) and total postoperative hospital stay (4 vs 5.5 days, P<0.001) [Table 2]. In multiple linear regression analysis, FT perioperative care was an independent predictor of shorter total postoperative hospital stay (P<0.001), whereas presence of complications (P<0.001), right-sided colonic resection (P=0.011), and male gender (P=0.046) predicted a longer hospital stay.

### Secondary outcome measures

The FT group was superior to the traditional group in all recovery parameters, including lower pain score, earlier return of gastrointestinal function, and shorter time to walk independently (Table 2). The overall 30-day complication rate also was lower in the FT than traditional group (14.1% vs 28.1%, P=0.051), as was the total direct cost (HK$96 897 vs HK$110 187, P=0.054). Quality of life at baseline was similar; all SF-36 subscales declined significantly at 2 weeks. At 12 weeks, physical functioning (P=0.002), bodily pain (P=0.018), social functioning (P=0.017), and role-emotional (P=0.033) were better in the FT than traditional group.

The preoperative level of cytokines and CRP was comparable between the two groups. IL-6 level peaked at 2 hours after surgery and was lower in the FT than traditional group (58.54 vs 74 pg/mL, P=0.05); the peak level of IL-1β and CRP was comparable between the two groups. The preoperative cell count of all the lymphocyte subsets was comparable between the two groups. After surgery, a significant depression of all the lymphocyte subsets over time was observed in both groups. Cell-mediated immune responses were less suppressed in the FT group on day 1 after surgery, as indicated by more circulating total T cells (P=0.009), helper T cells (P=0.011), and natural killer-like T cells (P=0.018).

## Discussion

Compared with patients who received traditional perioperative care, those who received FT perioperative care had a lower pain score, earlier return of gastrointestinal function, lower morbidity, better preservation of quality of life, and shorter total postoperative hospital stay. FT perioperative care was an independent predictor of shorter total postoperative hospital stay after laparoscopic colorectal surgery. It improved the benefits of laparoscopic colorectal surgery and reduced the duration of hospital stay without increasing the readmission rate. The total direct cost in the FT group was reduced by about HK$13 000 per patient as a result of lower hospitalisation cost and cost of managing complications. As laparoscopic colorectal surgery has a higher direct cost than open surgery, the cost savings of the FT perioperative care may help reduce the financial burden on the hospital/healthcare system and improve the cost-effectiveness of the procedure.[1]

Patients who underwent laparoscopic colorectal surgery and received FT perioperative care

had less stress response (lower interleukin-6 peak level) and better-preserved cell-mediated immunity. This was attributed to decreased preoperative fasting, administration of carbohydrate-loaded drink preoperatively, prevention of intraoperative hypothermia, and perioperative fluid restriction. FT perioperative care can maximise the immunological benefits of laparoscopic surgery for colorectal cancer that may have implications for tumour recurrence and long-term patient survival.[5]

Our study had several limitations. The study population represented a select group of patients who underwent uncomplicated elective laparoscopic resection of colonic or upper rectal cancer. Patients with mid or low rectal cancer and patients undergoing complex or combined laparoscopic procedures were excluded. These complicated cases are more likely to have a longer recovery time and higher morbidity, and it is uncertain whether FT perioperative care would be beneficial. Both study groups were admitted to the same surgical ward and cared for by the same team of surgical and nursing staff. This might have accounted for the intergroup crossover. Although the interventions were protocol-driven, a geographically separate ward for different study groups would be desirable to minimise performance bias and protocol contamination.

## Conclusion

Compared with traditional perioperative care, FT perioperative care for laparoscopic surgery for colorectal cancer resulted in faster clinical recovery, reduced morbidity, lower hospital cost, less stress response, and better-preserved cell-mediated immunity. FT perioperative care was an independent predictor of shorter total postoperative hospital stay.

## Funding

This study was supported by the Health and Health Services Research Fund, Food and Health Bureau, Hong Kong SAR Government (#08090061). The full report is available from the Health and Medical Research Fund website (https://rfs1.fhb.gov.hk/index.html).

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TABLE 2. Patient characteristics and outcome

| Parameter | Fast-track perioperative care (n=64) | Traditional perioperative care (n=64) | P value |
|---|---|---|---|
| No. of males:females | 40:24 | 39:25 | 0.856 |
| Age (years) | 65.3±8.7 | 68.2±8.4 | 0.060 |
| Body mass index (kg/m$^2$) | 22.7±3.3 | 23.3±3.2 | 0.239 |
| Patients with comorbidities | 33 (51.6) | 40 (62.5) | 0.211 |
| American Society of Anesthesiologists grade | | | 0.492 |
| I | 21 | 16 | |
| II | 38 | 40 | |
| III | 5 | 8 | |
| Tumour stage | | | 0.955 |
| pT0 | 11 | 13 | |
| pT1 | 6 | 6 | |
| pT2 | 6 | 4 | |
| pT3 | 35 | 36 | |
| pT4 | 6 | 5 | |
| Nodal stage | | | 0.827 |
| pN0 | 42 | 44 | |
| pN1 | 15 | 15 | |
| pN2 | 7 | 5 | |
| Types of surgery | | | 0.508 |
| Right hemicolectomy | 22 | 29 | |
| Left hemicolectomy | 7 | 6 | |
| Sigmoid colectomy | 18 | 18 | |
| Anterior resection | 17 | 11 | |
| Operative time (minutes) | 177.2±34.8 | 178.1±45.4 | 0.896 |
| Blood loss (mL) | 20 (0-200) | 20 (0-200) | 0.577 |
| Visual analogue scale pain score | | | |
| Day 1 | 3.8±2.0 | 5.1±1.9 | <0.001 |
| Day 2 | 2.6±1.3 | 3.6±1.8 | <0.001 |
| Day 3 | 2.2±1.2 | 3.0±1.5 | 0.001 |
| Time to first passing flatus (days) | 2.0±0.9 | 2.9±1.0 | <0.001 |
| Time to first bowel motion (days) | 3.2±1.3 | 4.4±1.5 | <0.001 |
| Time to resume normal diet (days) | 2.3±1.0 | 4.5±4.4 | <0.001 |
| Time to walk independently (days) | 2.1±0.8 | 3.8±2.8 | <0.001 |
| Postoperative hospital stay (days) | | | |
| Median (range) | 4 (2-24) | 5 (4-59) | <0.001 |
| Mean±SD | 4.7±2.9 | 7.8±8.3 | <0.001 |
| Readmission within 30 days of surgery | 4 (6.3) | 4 (6.3) | 1.000 |
| Total postoperative hospital stay (days) | | | |
| Median (range) | 4 (2-24) | 5.5 (4-59) | <0.001 |
| Mean±SD | 5.0±3.3 | 8.3±8.6 | <0.001 |
| Patients with complications | 9 (14.1) | 18 (28.1) | 0.051 |
| Reoperation | 1 (1.6) | 2 (3.1) | 1.000 |
| Operative death | 0 | 0 | |
| Total direct cost (HK$) | 96 897±18 157 | 110 187±51 651 | 0.054 |

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

# Parkinson disease and leucine-rich repeat kinase 2 gene mutation: abridged secondary publication

SL Ho *, PWL Ho, DCW Siu

KEY MESSAGES

1. Sporadic Parkinson disease (PD) involves a complex interaction of three major risk factors: genetic susceptibility, environmental toxicity, and ageing. We developed a new experimental protocol using a mouse model that carries a specific mutation at the same genetic location in parallel with the humans. We administered twice weekly oral doses of a naturally occurring pesticide (rotenone) over half the lifespan of the mouse to mimic chronic exposure to environmental toxicity. Ageing is inherent in the course of experiments. We found brain abnormalities and locomotor deficits in the new model are more faithful of the human PD than other existing models.
2. Although our LRRK2 knockin mice do not show obvious disease phenotype, they had greater reduction in striatal dopamine uptake, with locomotor deficits that were slower to recover than wild-type mice after reserpine injection (dopaminergic vesicle uptake blocker). These indicate that the mutant mice are more susceptible to striatal synaptic dysfunction even at young age, supporting its relevance in PD.
3. We cross-bred a new colony of mutant LRRK2 mice with fluorescent dopaminergic cells. This enables us to study, isolate, or image live dopaminergic neurons that degenerate in PD with greater ease and clarity. Our gene profiling studies showed significant differences in expression of several genes in nigrostriatal dopaminergic neurons of aged mutant mice.
4. The role of LRRK2 in phosphorylation of Rab GTPases proteins was explored using our LRRK2 mutant mice, including live mouse embryonic fibroblasts. This has important implications for the development of new treatments for PD such as LRRK2 inhibitors.
5. There is a lack of treatment modalities that can modify the progression of PD. Existing treatments relieve symptoms without altering its progression. It is still unclear how PD develops and progresses. Our model can help elucidate its complex disease processes and test therapeutic agents on these processes.

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## Introduction

Parkinson disease (PD) is a common neurodegenerative disease involving loss of dopaminergic neurons in substantia nigra par compacta, with nerve terminals projecting to the striatum. Progressive striatal dopamine depletion and synaptic dysfunction result in locomotor deficits. The disease process involves a complex interaction of ageing, genetic susceptibility, and environmental factors.

PD is rare before age 50 years and its occurrence increases substantially with ageing. Whole genome studies have linked several genes to sporadic PD. Farmers chronically exposed to environmental factors (eg pesticides) are more likely to develop PD. A naturally occurring pesticide, rotenone, is used in experimental models of PD. Current therapies of PD fail to modify disease progression. Hence, developing an appropriate experimental model involving these aetiological factors is crucial to explore the disease processes and develop therapeutic strategies.

Almost all existing experimental models of PD either use high doses of toxins given over a short period or use genetic models that either overexpress (transgenic) or have absent (knockout) gene expression. Although high acute doses of toxins can cause acute parkinsonian features, it does not reflect the situation in human PD that progresses gradually. Hence, chronic small doses of a toxin may better reflect the environmental toxicity.

Leucine-rich repeat kinase 2 (LRRK2) mutations are the commonest genetic risk in familial and sporadic PD. LRRK2-associated PD demonstrates similar clinical features as sporadic PD, suggesting parallel disease mechanisms. Although existing LRRK2 transgenic and knockout mice have parkinsonian features, they have abnormal LRRK2 expression which do not reflect human mutant

LRRK2 carriers who have similar LRRK2 expression levels as those without the mutation (wild-type [WT]). The key feature of our knockin mice is that the desired LRRK2$^{R1441G}$ mutant gene is integrated into a known intended locus in the genome. This is important because the mutated LRRK2 gene will achieve biological or natural expression patterns and levels as in WT. In contrast, the desired gene in the transgenic model is randomly integrated in the host genome under its own artificial promoter, leading to abnormally high expression levels with more robust phenotype. Although the transgenic model is useful in exploring gene function, the tradeoff is that transgenic model does not faithfully reflect the human disease. The gene expression pattern might be totally different from the native protein. For instance, overexpression of the LRRK2 gene in all brain regions in transgenic mice could lead to ectopic expression (ie, abnormally high LRRK2 expression in brain regions) and that could lead to a less accurate disease phenotype. It is noteworthy that non-symptomatic human carriers of the mutant LRRK2 gene have subtle abnormalities (elevated α-synuclein oligomer levels in cerebrospinal fluid, abnormal functional imaging in DAT-SPECT) but may not necessarily develop PD symptoms even in old age. Hence, our LRRK2 knockin mouse model can more accurately mimic human disease condition.

Mitochondrial dysfunction is a key feature in sporadic and familial PD. Accumulation of defective mitochondria and exposure to pesticides (eg rotenone) contribute to mitochondrial dysfunction. Mitochondria are key cellular organelles to supply energy by producing adenosine triphosphate (ATP). Nigrostriatal dopaminergic neurons are vulnerable to energy deficiency because of high energy-dependent activities (eg dopamine turnover) in their vast network of striatal presynaptic terminals. Hence, efficient mitochondrial ATP production in dopaminergic nerve terminals is crucial for survival. Pre-synaptic dysfunction in striatum is one of the earliest features of PD. We aim to develop a mutant LRRK2 mouse model of PD and investigate early disease processes (Fig. 1).

## Results

### To identify changes in mice with LRRK2$^{R1441G}$ mutation

We have successfully generated LRRK2 knockin mouse colony, back-crossed to WT C57BL/6 mice for over eight generations to ensure similar genetic background as WT, apart from the mutation (Fig. 2a). Although most mutant LRRK2 human carriers are heterozygous, we used the homozygous knockin mice to accentuate the genetic risk. There were no obvious physical differences between mutant and WT mice even in old age. The mutant mice were fertile and had normal body weight and brain size. Histological examination did not show obvious dopaminergic neuronal loss or striatal dopamine transporter protein expression. There were no observable differences in the striatal synaptic structure between young WT and mutant mice under electron microscopy.

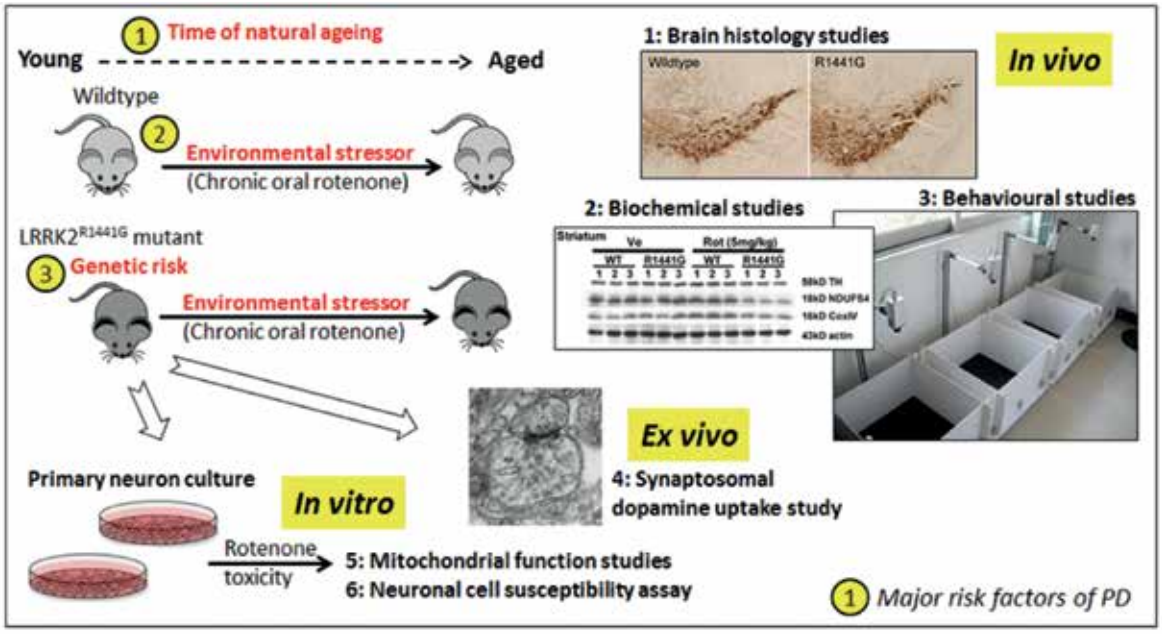


FIG 1. Overview of the current project scheme

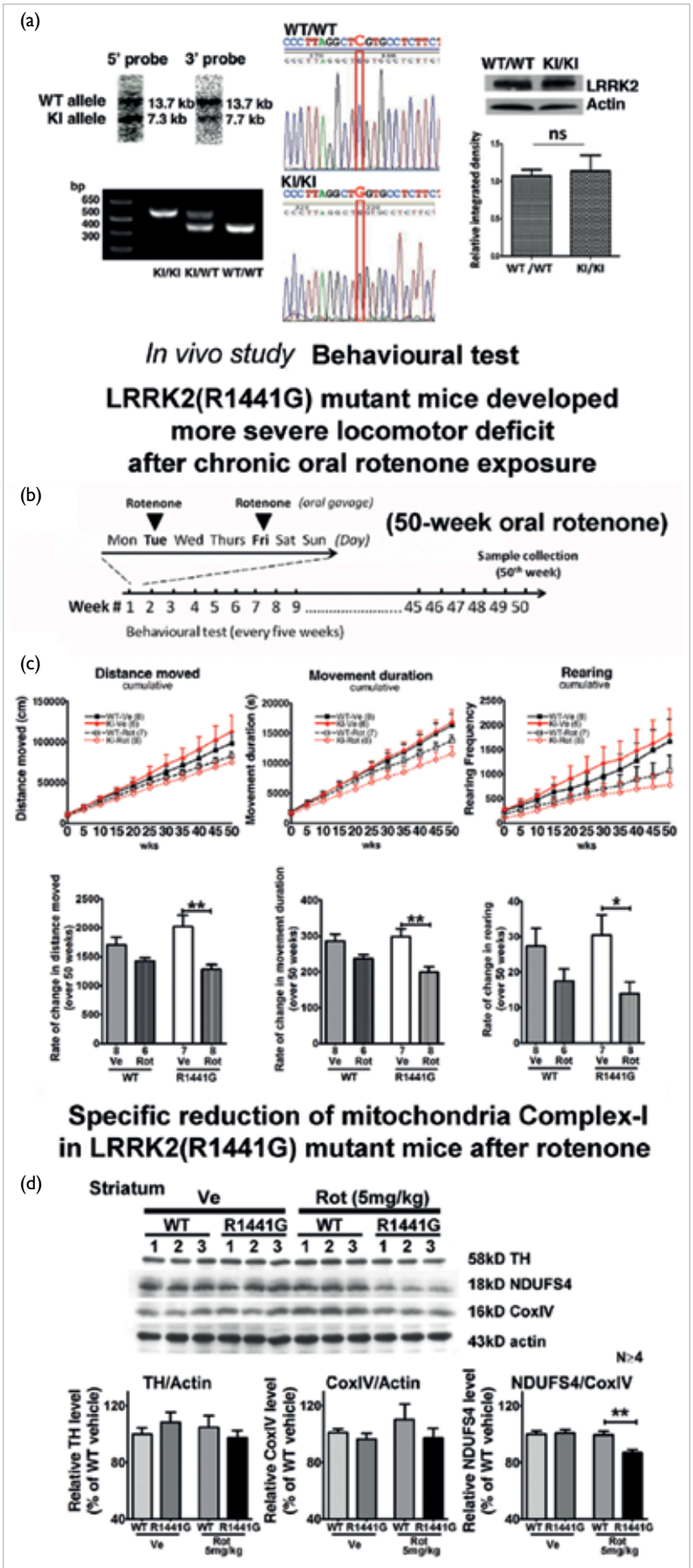


FIG 2. Successful generation of LRRK2$^{R1441G}$ knockin mice and key findings of the Behavioural tests after chronic oral rotenone administration

## To explore differences in neural signals in mutant mice

We explored striatal neurotransmission by measuring the local field potential using micro-electrode recording in vivo, stereotaxically positioned at dorsal striatum. Differences in amplitudes of the integrated neural signal between WT and mutant mice were assessed at three frequency bands: 3-8 Hz (theta), 13-30 Hz (beta), and 30-55 Hz (gamma). Our analyses showed no significant differences in the amplitudes of beta and gamma waves between young WT and mutant mice, although we observed a trend approaching significant difference for theta waves. These results need further evaluation on more mice, including aged mice. Although decreased amplitudes of theta waves are associated with anxiety, poor emotional awareness, and stress, the implications of these findings remain unclear. Interestingly, we observed that untreated mutant mice appeared more anxious than WT mice in the open-field behavioral test (by rearing frequency).

## To determine whether the mutation confers genetic susceptibility against environmental stress

After we stressed the young mice using a single reserpine injection to cause temporary synaptic dysfunction, mutant mice had greater reduction of striatal synaptosomal dopamine uptake with more severe locomotor deficit (ie, less distance moved, velocity, and movement duration). Locomotor activity also recovered slower in mutant mice than in WT mice. These results indicate that LRRK2 mutation conferred a genetic susceptibility to striatal pre-synaptic dysfunction even at a young age.

We then explored whether mutant LRRK2 mice were susceptible to rotenone. Primary cortical and mesencephalic dopaminergic neuronal cultures from mutant mice were more susceptible to rotenone than WT mice, with more cell death and less energy supply (ATP) [Fig. 3a-e]. As striatal dopamine uptake into presynaptic nerve terminals is highly energy-dependent, the effects of rotenone on this process was investigated. Using striatal isolates containing presynaptic terminals, mutant mice had significantly lower dopamine uptake in striatal synaptosomes after rotenone toxicity (Fig. 3f-g), indicating that LRRK2 mutation conferred a genetic susceptibility to ATP depletion at the dopaminergic nerve terminals in striatum.

We then studied the effects of a combination of ageing, genetic susceptibility, and environmental toxicity. Small doses of oral rotenone were administered twice weekly over 50 weeks to reflect chronic environmental toxicity (Fig. 2b). Reduced Complex-I level was found in PD patients. We used rotenone because it is a specific mitochondrial

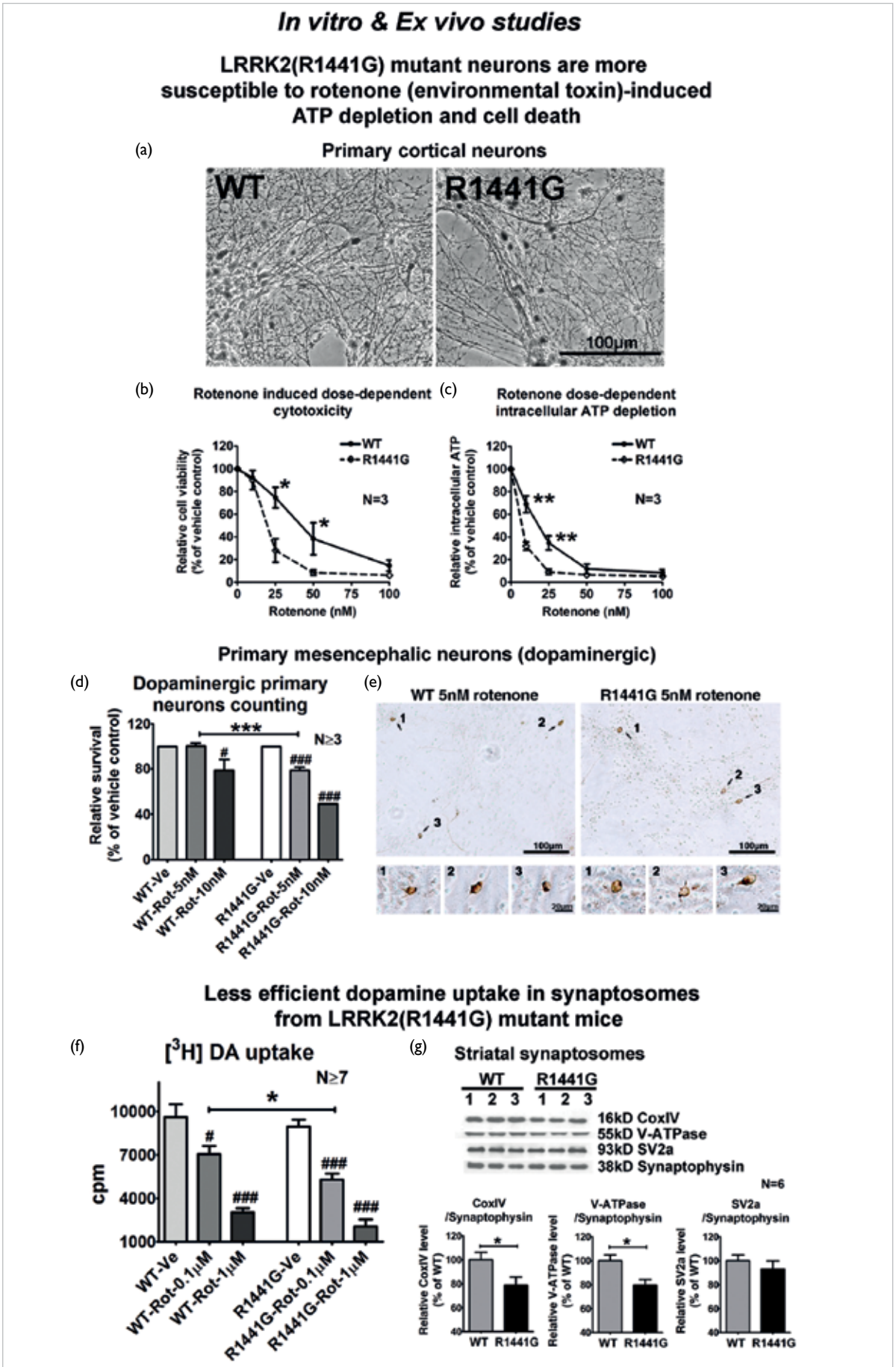


FIG 3. In vitro mitochondrial function studies and ex vivo dopamine uptake assay

Complex-I inhibitor that causes mitochondrial dysfunction. Ageing is inherent in this model because it was administered over half the normal lifespan of the mice.

Although the levels of apoptosis (programmed cell death) were similarly increased between mutant and WT mice after 50 weeks of oral rotenone, NDUFS4 (component of mitochondrial Complex-I) was significantly reduced in striatum of mutant mice, compared with WT mice, indicating that rotenone had a greater adverse impact on their mitochondria (Fig. 2d). We then assessed whether rotenone had any adverse effects on different aspects of locomotor activity of the mice by performing open-field tests every 5 weeks over 50 weeks. Interestingly, untreated mutant mice (ie, vehicle-treatment control) appeared more active than the WT mice, with greater cumulative distance moved and rearing frequency although the differences were not significant (Fig. 2c). Cumulative locomotor activity decreased in both WT and mutant mice treated with rotenone over 50 weeks. However, the decrease was significantly greater in mutant mice than WT mice. The cumulative distance moved, movement duration, and rearing frequency for rotenone-treated mutant mice over 50 weeks were significantly less than those for vehicle-treated mutant mice (Fig. 2c). However, rotenone-treated WT mice were not significantly affected by rotenone as compared with the vehicle-treated WT mice. These findings show that the LRRK2 mutation conferred a genetic susceptibility to rotenone toxicity.

### To identify changes in gene expression in dopaminergic neurons

In collaboration with RIKEN, Japan, we cross-bred our mutant LRRK2 mice with their transgenic mouse that expresses tyrosine hydroxylase-gene-promoter specific GFP[2] to obtain a new colony of mice with LRRK2 knockin mutation and GFP fluorescent dopaminergic neurons. This allows direct visualisation of live dopaminergic neurons without immuno-labelling. Using fluorescence-activated cell sorting, we obtained pure dopaminergic neurons from aged mice and found that at least four genes were differentially expressed in our mutant mice compared with the age-matched WT mice. As these are highly purified dopaminergic neurons from substantia nigra, these differences would be of great interest to explore disease-causing pathways.

## Significance and implications

The subtle abnormalities in the LRRK2$^{R1441G}$ knockin mice are much more akin to human mutant LRRK2 carriers than existing transgenic or knockout models. Our model combines three vital risk factors to developing sporadic PD (ageing, genetic susceptibility, and chronic oral rotenone). We found susceptibilities and abnormal changes in our mutant LRRK2 mice. This enables testing of potential therapeutic agents on these mice to modify disease progression.

## Funding

This study was supported by the Health and Medical Research Fund, Food and Health Bureau, Hong Kong SAR Government (#01120976), the Henry G Leong Professorship in Neurology, and the Donation Fund for Neurology Research. The full report is available from the Health and Medical Research Fund website (https://rfs1.fhb.gov.hk/index.html).

## Disclosure

The results of this research have been previously published in:

1. Ho PW, Leung CT, Liu H, et al. Age-dependent accumulation of oligomeric SNCA/ α-synuclein from impaired degradation in mutant LRRK2 knockin mouse model of Parkinson disease: role for therapeutic activation of chaperone-mediated autophagy (CMA). Autophagy 2020;16:347-70.
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# Adiponectin gene therapy for Alzheimer disease in a mouse model: abridged secondary publication

**RCL Ng, M Jian, LW Yick, M Bunting, JSC Kwan, SK Chung, KH Chan ***

**KEY MESSAGES**

1. Liver-specific expression of trimeric adiponectin can cross the blood-brain barrier.
2. Overexpression of trimeric adiponectin can improve memory function.
3. Overexpression of trimer adiponectin enhances neuronal insulin sensitivity and reduces amyloid-β deposition.
4. Adiponectin suppresses Aβ-induced microglia activation and neuroinflammatory responses, exerting protective effects to neurons and synapses in an Alzheimer disease mouse model.
5. Adiponectin deficiency exacerbates microglia-mediated neuroinflammation in an Alzheimer disease mouse model.

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## Introduction

Alzheimer disease (AD) is the most common cause (>60% cases) of dementia in older adults. Pathological studies of brains from AD patients have revealed extracellular accumulation of senile plaques containing amyloid-β fibrils in the neocortex and hippocampus. Amyloid-β peptides are derived from the cleavage of amyloid precursor protein. Amyloid-β-mediated toxicity includes oxidative stress secondary to excessive production of reactive oxygen species, activation of microglia and astrocytes that secrete proinflammatory cytokines by activating nuclear factor-kappa B (NF-κB) pathway, and synaptic dysfunction and loss resulting in cognitive impairments.

Adiponectin (APN) is a serum adipokine secreted predominantly by adipocytes, which possesses insulin-sensitising, anti-inflammatory, and anti-oxidative properties. Low-molecular-weight APN trimers, hexamers, and possibly globular APN are believed to be able cross the blood-brain barrier. We have reported that chronic APN deficiency in aged mice results in cerebral insulin resistance associated with AD-like pathologies and cognitive impairments.[1] Insulin-sensitising and anti-inflammatory effects of APN should be beneficial for AD characterised by cerebral insulin resistance, neuroinflammation, and oxidative stress. We hypothesise that APN is protective against amyloid-β-mediated neurodegeneration in AD. In this project, we investigated the therapeutic effects of trimeric APN on amyloid-β-mediated pathologies in a 5xFAD mouse model, using an adeno-associated virus (AAV)-mediated delivery method.

## Method

Mouse APN cDNA with FLAG epitope (Origene, USA) was subcloned into AAV vector (AAV2/8-eGFP) under control of apolipoprotein E promoter replacing eGFP sequence. Mutation in mouse APN gene C39S (cysteine to serine) ensured that only trimeric APN was produced by the transduced cells. The vector was packaged by triple transfection of HEK293 cells with either pAAV2/8-eGFP or pAAV2/8-APN-Flag, p5E18-VD2/8, and adenoviral helper plasmid pXX6. Vector particles were purified from cell lysate by polyethaleneglycol-ammonium sulphate method.

Female transgenic mice (5xFAD) carrying overexpress mutant human amyloid precursor protein and human PS1 harbouring were used as the animal model of AD. 5xFAD mice aged 4 months received AAV-APN gene therapy via intravenous injection (via tail vein) with purified AAV at a dosage of $1 \times 10^{11}$ vector genomes. Four months after AAV injection. Behavioural tests including Morris water maze test and openfield test were performed to investigate the spatial learning and memory functions as well as anxiety levels (Fig. 1a). Each group contained at least 10 mice for behavioural test. Mice were then dissected, and the brain, plasma, and liver collected.

For immunohistochemistry, brain sections (frontal cortex) were studied for amyloid-β with antibodies against amyloid-β residues 17-24 (clone 4G8), microglia with Iba1 antibody, and astrocytic

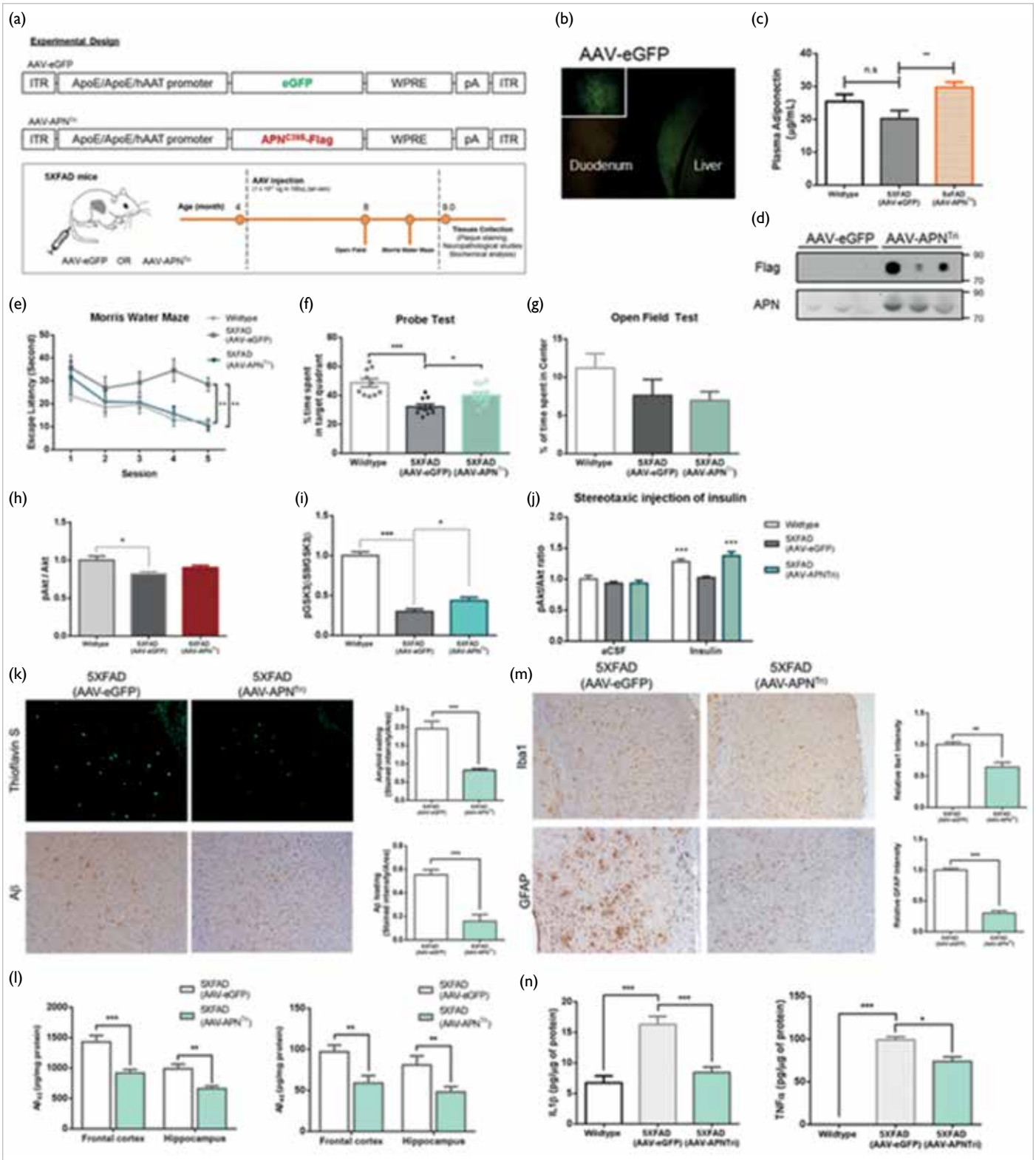


**FIG 1. Overexpressing trimeric adiponectin by liver-specific adeno-associated virus (AAV) delivery improves memory functions and Alzheimer disease (AD) pathologies in an AD mouse model.** (a) Schematic of experimental design. (b) Representative image showing eGFP expressed in the liver after AAV-eGFP delivery. (c) ELISA analysis of plasma adiponectin. (d) Western blot analysis of plasma trimeric adiponectin. (e) Escape latency of Morris water maze test. (f) Percentage of time spent in the target quadrant of the probe test in the Morris water maze. (g) Percentage of time spent in the centre region of the open field test. Densitometric analysis of Western blotting on the (h) pAkt/Akt and (i) pGSK3β/GSK3β in the hippocampus of the mice and on the (j) pAkt/Akt ratio after stereotaxic injection. (k) Immunostaining of amyloidβ (Aβ) using thioflavin S and anti-Aβ antibodies. (l) ELISA analysis of Aβ40 and Aβ42 in the frontal cortex and hippocampus. (m) Immunohistochemistry analysis of Iba1 and GFAP. (n) ELISA analysis of TNFα and IL1β Aβ42 in the frontal cortex and hippocampus.

activation with GFAP antibody. Thioflavin S fluorescent staining was performed to further confirm amyloid deposition. For immunoblotting, tissues lysates or cell lysates were studied by standard SDS-PAGE and western blotting for detecting proteins of interest using primary antibodies and secondary antibodies. Chemiluminescent signal was developed using Westernbright Quantum HRP substrate.

To study hippocampal insulin sensitivity, mice with AAV injections after 8 weeks were anaesthetised and subjected to intracerebral injection of insulin. Human insulin (0.02 IU/g bodyweight of mice) was injected to the right hippocampus by stereotaxic injection platform. The hippocampi were dissected 30 minutes after injection, and protein lysate collected for pAkt detection.

Murine BV2 microglia cell line and HT-22 hippocampal neuronal cells were used. BV2 cells and HT-22 cells were cultured in Dulbecco modified Eagle's medium with 10% foetal bovine serum and 1% penicillin/streptomycin. The cells were grown in a humidified incubator at 37°C with 5% $CO_2$. BV2 cells were pretreated with APN (10 μg/mL) or compound C (10 μM) for 2 hours and then treated with amyloid-β Oligomer (AβO) [10 μM] for 24 h in serum-free culture medium. To knockdown APN receptors in BV2 microglia, AdipoR1 and AdipoR2 siRNAs and non-targeting control siRNA were transfected to BV2 cells.

Cell viability were performed after co-culturing BV2 and HT22 cells using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide. The absorbance of the solution in each well was determined at 570 nm using a CLARIO star microplate reader. Amyloid-β40 and amyloid-$\beta_{42}$ in the soluble brain lysates were measured using the human amyloid-β40 and human amyloid-$\beta_{42}$, respectively. IL1β and TNFα levels in the brain lysates were measured using ELISA. The assays were performed following supplier instructions. The optical density of each well at 450 nm was determined by a CLARIO star microplate reader.

Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software) or SPSS (Windows version 24; IBM Corp, Armonk [NY], US). For Morris water maze test, dataset was analysed by two-way ANOVA. Open field test was analysed by one-way ANOVA. In other experiments, between-group differences were determined with one-way ANOVA, followed by Bonferroni post hoc test. Alternatively, the mean significant difference between two groups was determined with two-tailed unpaired Student t test. Statistical significance was defined as $P<0.05$.

## Results

GFP signal was detected in the liver but not in other tissues 1 month after injecting AAV-eGFP virus (Fig. 1b). ELISA analysis indicated that the plasma APN level was significantly increased in 5xFAD mice injected with AAV-APN$^{Tri}$ APN 2 months after injection ($P<0.01$, Fig. 1c). Western blot analysis by anti-flag and anti-APN antibodies indicated that trimeric APN (~74 kDa) level was increased in plasma (Fig. 1d).

In the Morris water maze test, AAV-APN$^{Tri}$-treated 5xFAD mice showed shorter escape latency significantly compared with that of AAV-eGFP-treated 5xFAD mice, and the escape latency was comparable to that of the wildtype littermates (Fig. 1e). In the probe test, 5xFAD mice injected with AAV-APN$^{Tri}$ spent significantly more time in the target quadrant than control 5xFAD mice (Fig. 1f). In the open field test, treatment with AAV-APN$^{Tri}$ could not reduce the anxiety level in 5xFAD mice (Fig. 1g). These results demonstrated that overexpressing trimeric APN can improve memory and spatial learning performance but not psychiatric symptoms in the AD mouse model.

Western blot analysis demonstrated that AAV-APN$^{Tri}$ treatment insignificantly increased hippocampal Akt phosphorylation and moderately increased GSK3β$^{S9}$ phosphorylation (Figs. 1h & 1i). Phosphorylation of Akt in wildtype mice was increased after insulin injection, whereas that of AAV-eGFP-treated 5xFAD mice showed no significant change. However, the induction of Akt phosphorylation was rescued in 5xFAD with AAV-APN$^{Tri}$ treatment indicating that trimeric APN restores insulin sensitivity in the hippocampus of 5xFAD mice (Fig. 1j).

Next, we examined the effect of AAV-APN$^{Tri}$ on amyloid-β levels in the 5xFAD mice. AAV-APN$^{Tri}$ displayed significant reduction of amyloid and amyloid-β deposition in thioflavin S staining (Fig. 1k) and immunohistochemistry analysis (Fig. 1m). ELISA analysis also supported that amyloid-β40 and amyloid-β42 peptides were significantly reduced in the frontal cortex and hippocampus of 5xFAD mice after treating with AAV-APN$^{Tri}$ (Fig. 1l).

Iba1 levels in the frontal cortex of 5xFAD mice were reduced by >40% with AAV-APN$^{Tri}$ treatment. Immunohistochemistry staining of GFAP in the frontal cortex showed AAV-APN$^{Tri}$ treatment dramatically reduced GFAP levels by ~70% indicating reduction of astrogliosis. Reactivated microglia secretes proinflammatory cytokines in AD brains causing neuroinflammation. Both the levels of TNFα and IL1β increased in the cortex and hippocampus of 5xFAD mice after AAV-APN$^{Tri}$ treatment (Fig. 1n). These results indicated that trimeric APN treatment could suppress the amyloid-β-mediate neuroinflammatory responses in the AD mouse model.

We then studied if APN directly inhibits microglia activation and the molecular mechanism of

suppressing the cytokines secretion under amyloid-β stimulation (Fig. 2). AdipoR1 and AdipoR2 were found in BV2 microglia cell line and microglia cell in vivo. Reverse transcriptional PCR and western blot analysis were performed to detect mRNA and proteins, respectively. AβO induced the release of TNFα and IL-1β from BV2 cells, whereas APN inhibited the release of TNFα and IL-1β. In addition, $pAMPK^{T172}$ level was decreased in AβO-treated BV2 cells. In contrast, pretreating APN rescued the reduction of $pAMPK^{T172}$ level upon AβO-treatment. NF-κB p65 level in nuclear extracts was markedly increased in AβO treated BV2 cells, whereas APN pretreatment significantly reduced nuclear NF-κB p65 level. These results were further supported by the addition of compound C, which blocked AMPK phosphorylation. Together, these data suggest that APN inhibits AβO-induced proinflammatory cytokines through AMPK- NF-κB signalling cascade in microglia.

AdipoR1 and AdipoR2 expressions were significantly inhibited by siRNA at the dose of 100 nM. The ability of APN to suppress proinflammatory cytokines TNFα and IL-1β secretion from AβO-treated BV2 cells was abolished by AdipoR1 siRNA-transfections, but not by AdipoR2 siRNA-transfection. These data indicate that APN suppressed AβO-induced microglial proinflammatory cytokines release through AdipoR1.

To further investigate if APN could protect against neuronal toxicity induced by AβO-activated microglia, we used a transwell system to co-culture BV2 cells and HT-22 cells. The viability of HT-22 hippocampal cells co-cultured with BV2 microglia cells treated with AβO and APN was significantly increased. The presence of compound C blocked the neuroprotective effect of APN. Altogether, these results further reinforce that APN protects neuronal survival by suppressing microglia activation through AMPK activation.

We found that lacking APN increased cortical and hippocampal TNFα and IL1β levels, and that APN deficiency increased microgliosis in the cortex and hippocampus with the increased levels of microglia associated TNFα and IL1β expression (Fig. 3). These results suggest that APN deficiency results in increased activation and neuroinflammatory response of microglia in AD.

## Discussion

We demonstrated the therapeutic potential of overexpressing trimeric APN using the AAV delivery method. Trimeric APN expressed in the liver cells can raise the trimeric APN level in plasma. By crossing the blood-brain barrier, trimeric APN improves memory functions and reduce amyloid-β deposition. Most importantly, we demonstrated the insulin-sensitising and anti-inflammatory effects of trimeric APN to central nervous system. Trimeric APN showed potent effects to treat AD.

One study reported the therapeutic effects of osmotin, plant APN homologue, to treat AD in the amyloid transgenic mouse model. Similarly, daily injection of osmotin can reduce amyloid pathologies by inhibiting the level of β-secretase, an enzyme which generates amyloid-β. AD mice treated with osmotin also showed improved neuronal functions and restored dendritic spine density in the hippocampus and reversed cognitive impairments.[2,3] Application of osmotin and trimeric APN demonstrated great potential to treat AD by activating APN signalling and its downstream effectors.[4] We further provide evidence to support activating APN signalling in central nervous system can reduce neuroinflammatory responses. Our method also demonstrated better clinical application with single injection (with effects last for months), compared with daily osmotin injection.

Overexpression of neurotrophic factors, hormones, or growth factors demonstrated great promises to protect neurons. However, injection of recombinant proteins is costly. Most of them such as brain-derived neurotrophic factor and glia-derived neurotrophic factor are not blood-brain barrier permeable requiring intracranial injection of AAV to overexpress these factors.[5] Intracerebral injection is an invasive method and may lead to traumatic brain injury. Although these factors have therapeutic potential, the method is still not applicable to treat neurodegenerative disease owing to the invasive drawbacks. Liver-specific AAV transduction can overexpress target protein to treat neurodegenerative disease. This method can be applied to overexpress other growth factors such as FGF21, which can cross the blood-brain barrier and has been recognised as neuroprotective agent.

Further investigation should include injection of AAV-APNTri to transgenic $Tau^{P301L}$ mice or 3xTg mice to determine the efficacy of APN in suppressing tau-mediated pathologies, and generation of AD patient-specific iPSC-derived neurons to determine the therapeutic efficacy of human trimeric APN to human neurons. Moreover, before clinical trials, we should generate $AAV\text{-}APN^{Tri}$ (carrying human APN sequence) vector and proceed to large production of $AAV\text{-}hAPN^{Tri}$ particles.

## Conclusion

AAV-trimeric-APN can reverse memory impairments and improve neuropathology in AD mice. Trimeric APN reduces amyloid-β deposition but the mechanism has not been identified yet in this study. Most importantly, trimeric APN exerts neuroinflammatory effects by inhibiting microglia reactivation but not enhances neuronal insulin

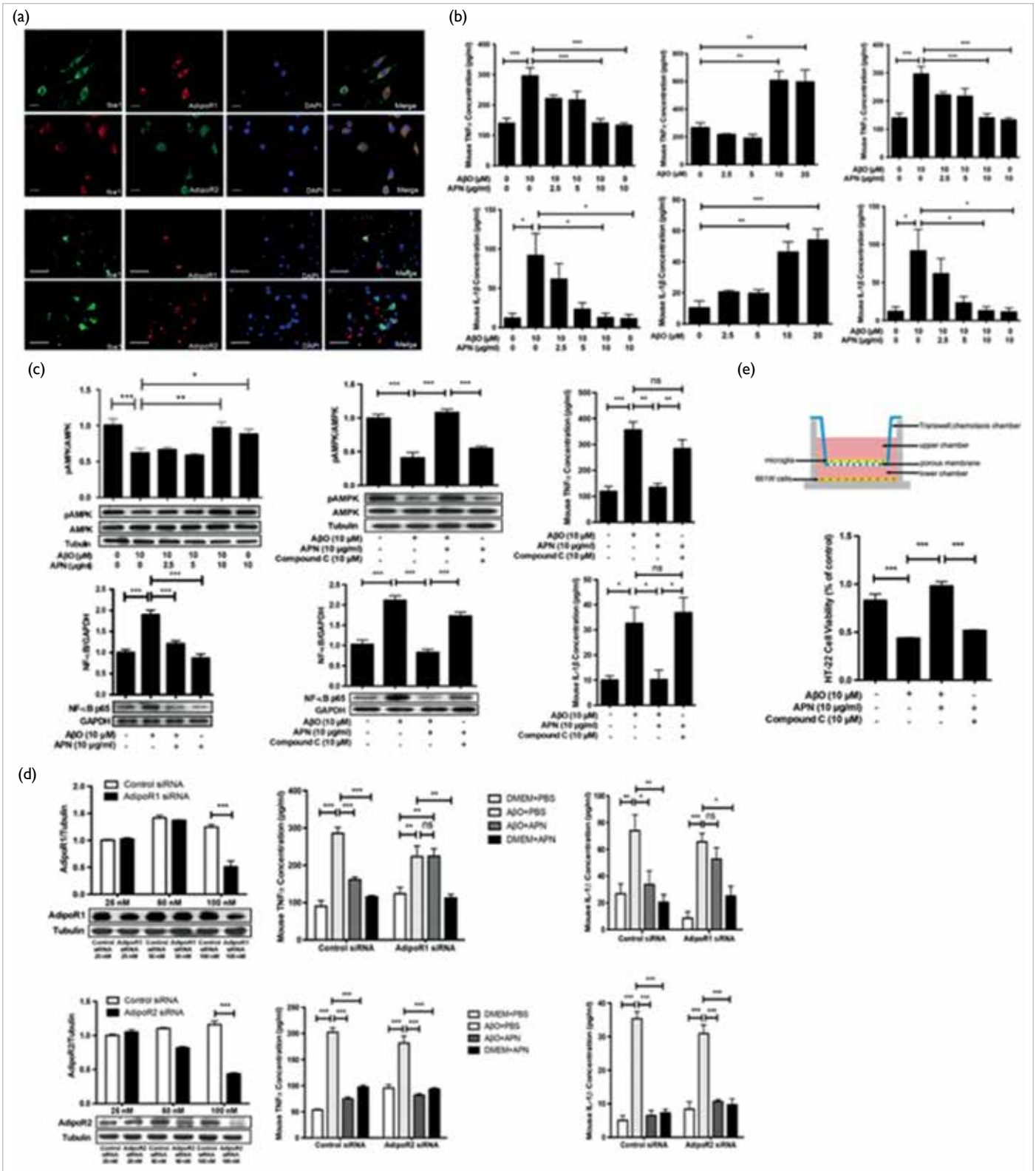


**FIG 2. Adiponectin (APN) suppresses amyloid-β (Aβ)-induced microglia activation and proinflammatory cytokines secretion to protect neurons from neuroinflammatory toxicity through AdipoR1-AMPK-NF-κB signalling cascade.** (a) Immunofluorescent staining of AdipoR1 and AdipoR2 in the BV2 cells and microglia in mice brain. (b) ELISA assay of TNFα and IL1β of medium after AβO stimulation with or without APN pretreatment. (c) Western blot analysis of pAMPK/AMPK and nuclear NF-κB levels in the AβO-exosed BV2 cells with or withour APN and compound C pretreatment. (d) ELISA assay of TNFα and IL1β of medium in the adipoR1- and adipoR2-knockdown BV2 cells exposed to AβO stimulation with or without APN preteatment. (e) MTT assay and survival analysis after co-culturing BV2 and HT22 cells.

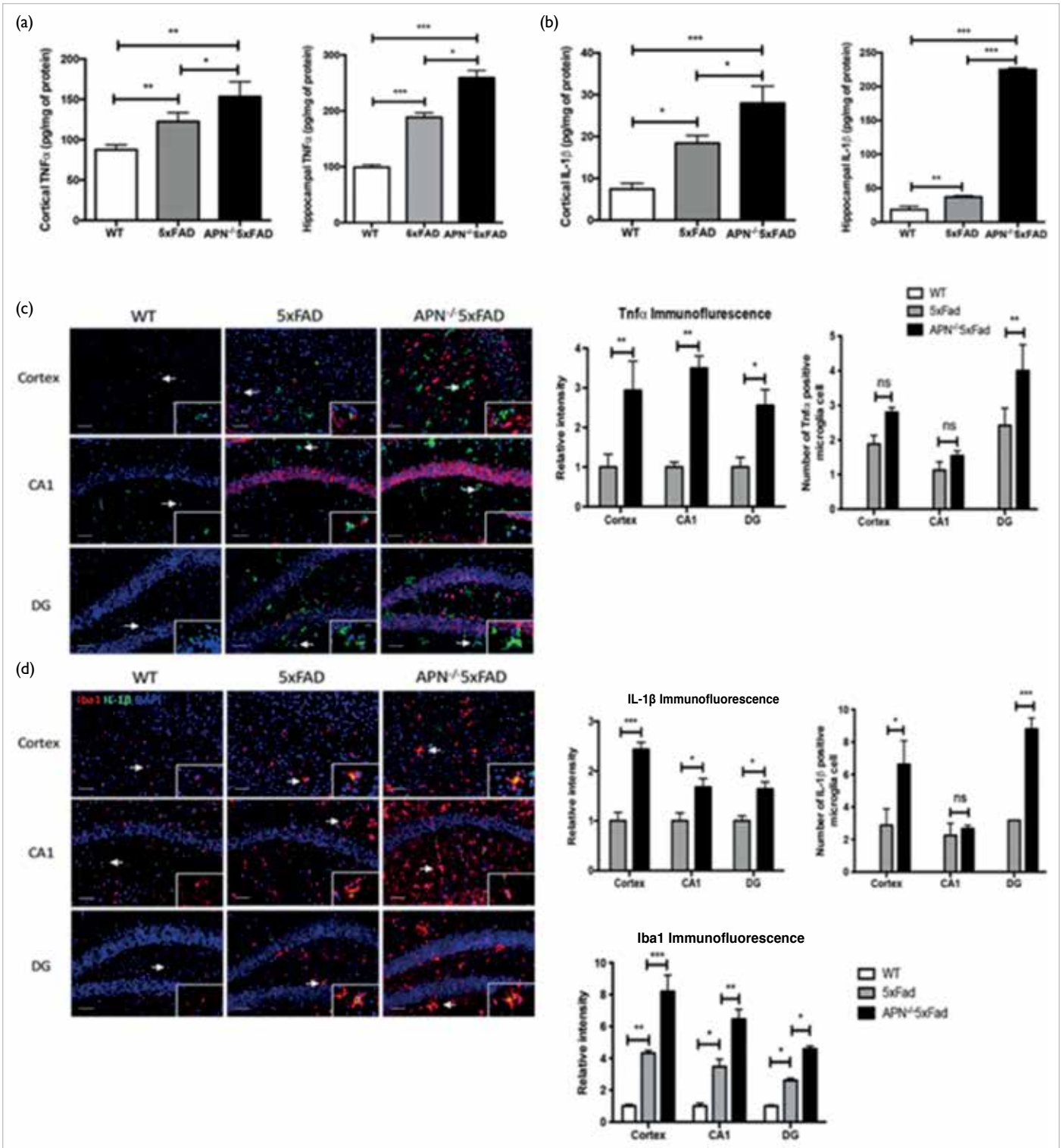


**FIG 3. APN deficiency increases microgliosis and proinflammatory cytokines expression in the cortex and hippocampus in 5xFAD mice.** (a & b) ELISA analysis of TNFα and IL1β levels in the cortex and hippocampus. (c & d) Double immunofluorescent staining of TNFα or IL1β and Iba1β in the cortex and hippocampus.

sensitivity. It suppresses microglial activation through AdipoR1/AMPK/NF-κB signalling. Reduction of APN in 5xFAD mice increases microglial activation along with dramatic elevation of TNFα and IL1β levels in both the cortex and hippocampus. These support the hypothesis that APN is essential to regulate microglia activation, and trimeric APN has potential therapeutic effects to treat AD.

## Funding

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## Disclosure

The results of this research have been previously published in:

1. Jian M, Kwan JS, Bunting M, Ng RC, Chan KH. Adiponectin suppresses amyloid-β oligomer (AβO)-induced inflammatory response of microglia via AdipoR1-AMPK-NF-κB signaling pathway. J Neuroinflammation 2019;16:110.

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# Huperzine A in treatment of amyloid-β-associated neuropathology in a mouse model of Alzheimer disease: abridged secondary publication

**Q Yuan *, ZX Lin, W Wu, WN Albert, BCY Zee**

KEY MESSAGES

1. Huperzine A treatment resulted in a reduction of acetylcholinesterase activity in TgCRND8 Alzheimer disease mice.
2. Huperzine A, an acetylcholinesterase inhibitor for Alzheimer disease, could not inhibit GSK3β activity, and therefore did not facilitate prevention of amyloid precursor protein processing/amyloid-β generation in TgCRND8 mice.

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## Introduction

Huperzine A is a novel *Lycopodium* alkaloid extracted from traditional Chinese herb *Huperzia serrata* (Thunb) Trev (Qian Ceng Ta). It is a potent selective reversible acetylcholinesterase (AChE) inhibitor and has been used in China for the treatment of Alzheimer disease (AD) since 1996.[1-3] Large, randomised, placebo-controlled, double-blinded clinical trials revealed that huperzine A can enhance memory, cognitive skills, and daily life abilities of patients with AD.[1] Clinical trials of huperzine A for treatment of age-related memory deficiency have been conducted in the United States.[3] New mechanisms of action for huperzine A have been discovered. In addition to its AChE inhibitory effect, huperzine A influences amyloid precursor protein (APP) processing to reduce the formation of amyloid-β (Aβ) peptides.[3] However, there has been no sufficient experimental evidence from AD animal models to elucidate the precise molecular mechanisms of huperzine A on APP processing. This study aimed to investigate whether huperzine A inhibited GSK3β, thereby facilitated prevention of APP processing/Aβ generation in TgCRND8 mice.

## Methods

This study was conducted from June 2015 to November 2017. Male TgCRND8 mice aged 5 months were randomly assigned to the treated or untreated group. The treated group received huperzine A dissolved in a vehicle of normal saline daily by intraperitoneal injection (0.80 μmol/kg). The untreated group received an equal volume of normal saline as a vehicle control. Treatment continued for 8 weeks, and then the mice were sacrificed by decapitation, and one hemisphere of each brain was processed for Aβ, IBA-1, and GFAP immunohistochemistry. The other hemispheres of the brains were processed for GSK3β, BACE1, CTF-APP, and B-actin Western blotting analysis.

Cross-sections of the brain were treated according to standard procedures. Briefly, the sections were incubated overnight at room temperature with the primary antibodies against Aβ (mouse, 1:3000, Sigma-Aldrich), IBA-1 (rabbit, 1:2000, Wako), and GFAP (mouse, 1:3000, Sigma-Aldrich) in 0.1 M PBS (pH 7.4) containing 10% normal goat serum and 0.2% Triton X-100. Then, antigens were visualised using Alexa 568-conjugated secondary antibody (1:800; Invitrogen). Finally, the sections were visualised under a fluorescence microscope (Zeiss).

To determine the brain cortex expression of GSK3β and its phosphorylation at serine 9, BACE1, and CTF-APP, tissue samples were homogenised in 0.1 ml lysis buffer reagent and serine protease inhibitor PMSF (both from Sigma-Aldrich) and centrifuged at 14,000 ×g for 30 min at 4°C. The supernatant was collected and total protein was measured using a protein assay (Bio-Rad). An equal volume of 2× sample buffer (100 mM Tris–HCl pH 6.8, 2.5% SDS, 20% glycerol, 0.006% bromophenol blue and 10% β-mercaptoethanol) was added to 30 μg total proteins. The samples were boiled and then electrophoresed in a 10%-15% SDS-polyacrylamide gel (Sigma-Aldrich) and transferred to a Hybond-P membrane (Amersham Bioscience).The blotted membrane was then incubated overnight with 5% skim milk in T-TBS (containing 0.1% v/v Tween 20). All antibody applications were done in T-TBS.

After the membranes were washed with T-TBS, they were incubated overnight at room temperature with GSK3β (rabbit, 1:3000, Cell Signaling Technology), GSK3β phosphorylated at Ser9 (rabbit, 1:3000, Cell Signaling Technology), BACE-1 (rabbit, 1:1000; Abcam), the C-terminal anti-APP antibody CT15 for full-length APP and CTF-β (rabbit, 1:2000, Cell Signaling Technology). The membranes were extensively washed with T-TBS and incubated for 1 hour with the secondary antibody (anti-mouse or anti-rabbit IgG peroxidase-conjugated antibody, 1:5000) [Sigma-Aldrich]. After washing, the proteins were detected using an ECL-Plus Western blotting detection system (GE Healthcare).

In accordance with methods described in our previous study,[4] brains were sectioned in 30 μm thickness using a microtome. Plaque deposition levels were examined in cortex. Images of 100× magnification were captured using a Zeiss microscope equipped with a SPOT camera and SPOT software (RT Color Diagnostic Instrument) on four sections per animal. By using ImageJ software, pictures were binarised to 8-bit black and white pictures and a fixed intensity threshold was applied to define the immunofluorescence staining. Measurements were performed for a percentage area covered by Bam-10, IBA-1, or GFAP immunostaining.

## Results

### Huperzine A treatment resulted in a reduction of AChE activity in the TgCRND8 mice

We examined whether huperzine A could inhibit AChE activity in TgCRND8 mice brain. To measure AChE activity, the brain cortex was added into 1% Tris-HCl buffer and homogenised. Homogenates were centrifuged at 3500 rpm for 10 minutes at 4°C. The supernatant was used as AChE enzyme source and stored at -80°C. AChE activity was measured using an Amplite Colorimetric AChE Assay Kit (AAT Bioquest). The AChE inhibitory activity observed in the control was considered to be 100%. Huperzine A inhibited AChE activity (Fig. 1a).

### Huperzine A treatment did not reduce Aβ plaque burden in the brain cortex of TgCRND8 mice

Aβ plaque immunostaining with the bam10 antibody and thioflavin S staining in TgCRND8 mice showed marked Aβ deposits in the cortex of TgCRND8 mice. Quantification of the Aβ immunoreactivity showed no significant reduction of plaque burden in huperzine A–treated animals compared with controls (Fig. 1b).

### Huperzine A treatment did not ameliorate Aβ-associated reactive gliosis or astrocytosis in the brain cortex of TgCRND8 mice

Microgliosis and astrocytosis in TgCRND8 mice were elevated phenotypically as a consequence of amyloid deposition. The degree of microgliosis as evaluated by IBA-1 load in the brain cortex was significantly amplified in vehicle-treated TgCRND8 mice relative to wildtype mice (data not shown), whereas it was not significantly reduced in huperzine A–treated TgCRND8 mice relative to vehicle-treated TgCRND8 mice (Fig. 2). Likewise, the magnitude of astrocytosis as assessed by clusters of GFAP-immunoreactive astrocytes (GFAP burden) was not significantly reduced in huperzine A–treated TgCRND8 mice, relative to vehicle-treated TgCRND8 mice (Fig. 2).

### Huperzine A treatment did not alter GSK3β activity or APP processing in TgCRND8 mice

We examined the levels of phosphorylated GSK3β, BACE-1, CTF-beta in the mice cortex. It is known that GSK3β is inhibited when Ser9 is phosphorylated.[5] However, we found no significant alteration of the inactive form of GSK3β phosphorylated at Ser9. The data suggest that huperzine A treatment did not alter GSK3β activity. Likewise, no remarkable alterations of BACE1 and CTF-β were observed in the brains of the huperzine A–treated TgCRND8 mice compared with vehicle-treated TgCRND8 controls (Fig. 3).

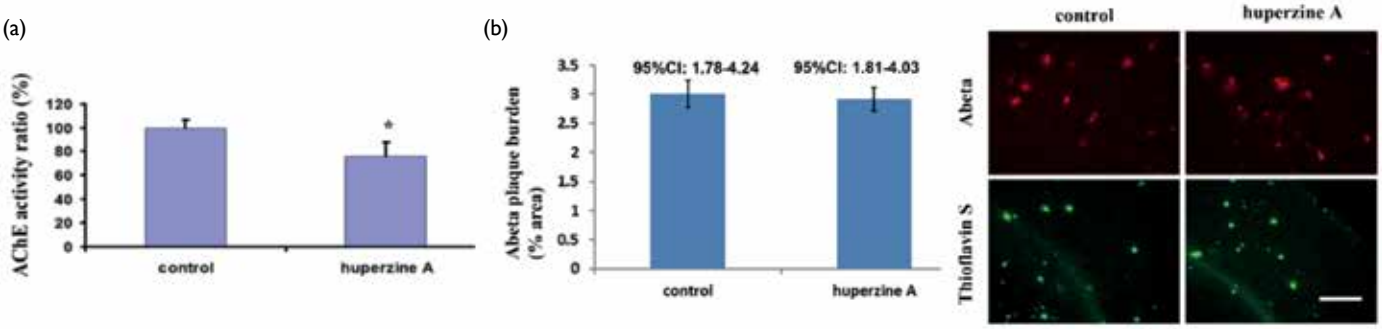


FIG 1. Effects of huperzine A treatment in the cortex of TgCRND8 mice on (a) acetylcholinesterase (AChE) activity and (b) amyloid-β plaque burden (as revealed by Thioflavin S staining and amyloid-β immunohistochemical staining).

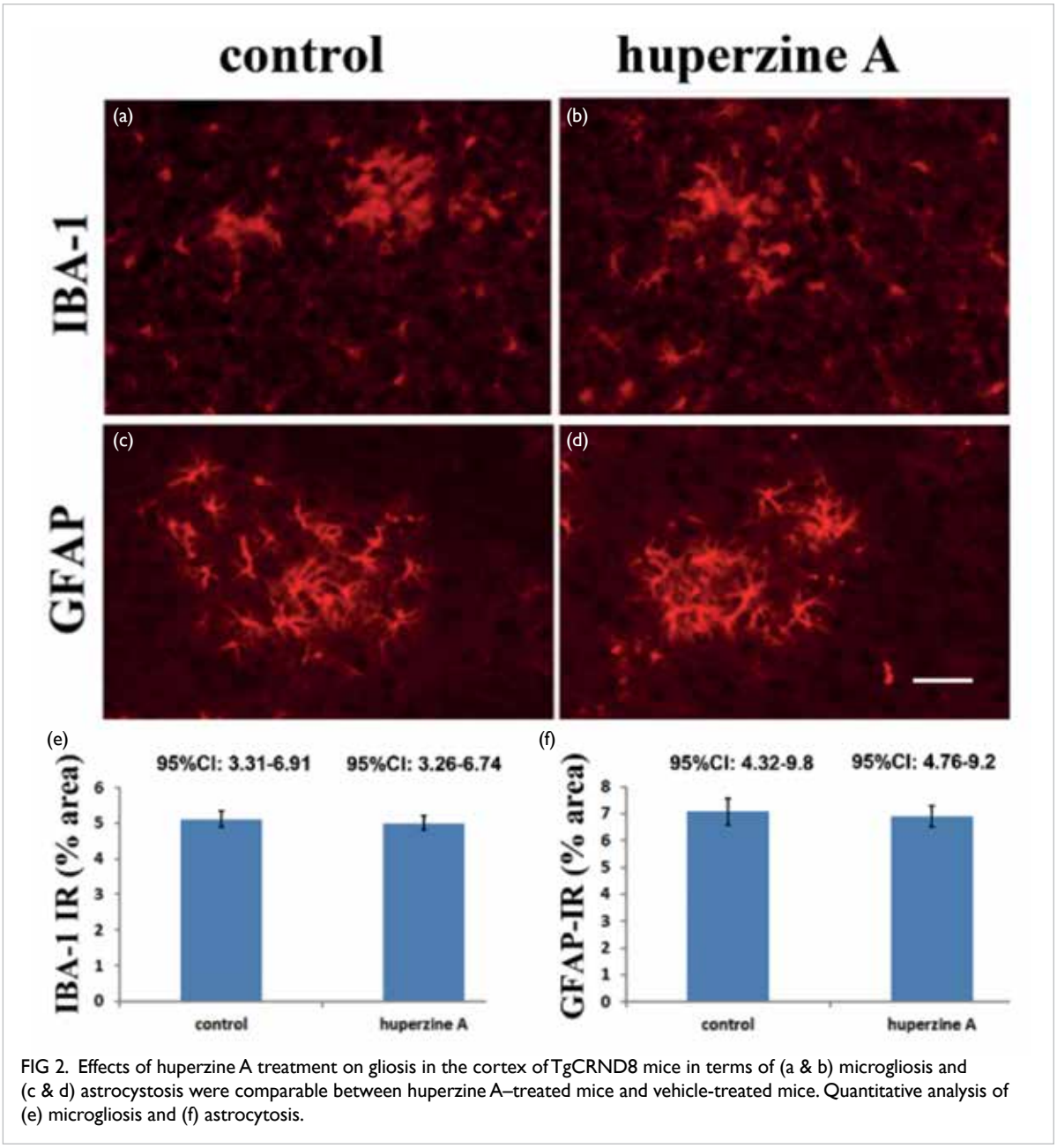


FIG 2. Effects of huperzine A treatment on gliosis in the cortex of TgCRND8 mice in terms of (a & b) microgliosis and (c & d) astrocystosis were comparable between huperzine A–treated mice and vehicle-treated mice. Quantitative analysis of (e) microgliosis and (f) astrocytosis.

## Discussion

Although huperzine A is a therapeutic drug for AD by inhibiting AchE activity in patients with AD, the actual therapeutic role of huperzine A in Aβ neuropathology has not been fully evaluated. Data from this study suggest that regular administration of huperzine A may not involve the mechanisms targeting amyloidogenic APP cleavage pathway observed in TgCRND8 mice with an early-onset AD-like pathology. Our results suggest that huperzine A has no beneficial effects in Aβ neuropathology of AD. First, no significant effects on brain Aβ plaque burden and associated gliosis were found in the TgCRND8 mouse model of AD. Second, Huperzine A did not significantly reduce CTFs and BACE-1, a key enzyme for APP cleavage. Third, huperzine A did not significantly inhibit GSK3B activity in the brain of TgCRND8 mice. It has been shown that GSK3β activity increases in cells expressing Swedish APP mutation and in AD presenilin-1 and presenilin-2 mutation lymphoblast cells via inactive Ser9 phosphorylated GSK3β. Studies have shown that GSK3β affects APP processing by modulating BACE-1 activity, thereby facilitating Aβ production, reinforcing that GSK3β plays a key role in APP processing/Aβ generation. Our findings that huperzine A did not block GSK3β activity in the brain of TgCRND8 mice may explain

its effect on Aβ plaque burden and associated gliosis. Huperzine did not inhibit GSK3β activity and modulate BACE-1 activity, thereby failed to facilitate APP processing/Aβ production. To exclude the possibility that huperzine A we used may be invalid, we assessed its effect on AchE activity. Huperzine A used in this study inhibited AchE activity in the brain of TgCRND8 mice. These findings suggest that the effect of huperzine A in preventing Aβ neuropathology needs further studies to confirm.

## Conclusions

Huperzine A, an AChE inhibitor for AD, could not inhibit GSK3β activity, and therefore did not facilitate prevention of APP processing/Aβ generation in TgCRND8 mice. Furthermore, huperzine A treatment did not inhibit Aβ-associated gliosis in TgCRND8 mice. The neuroprotective effect of huperzine A may need more studies to investigate the mechanisms involving targeting amyloidogenic APP cleavage pathway in AD treatment.

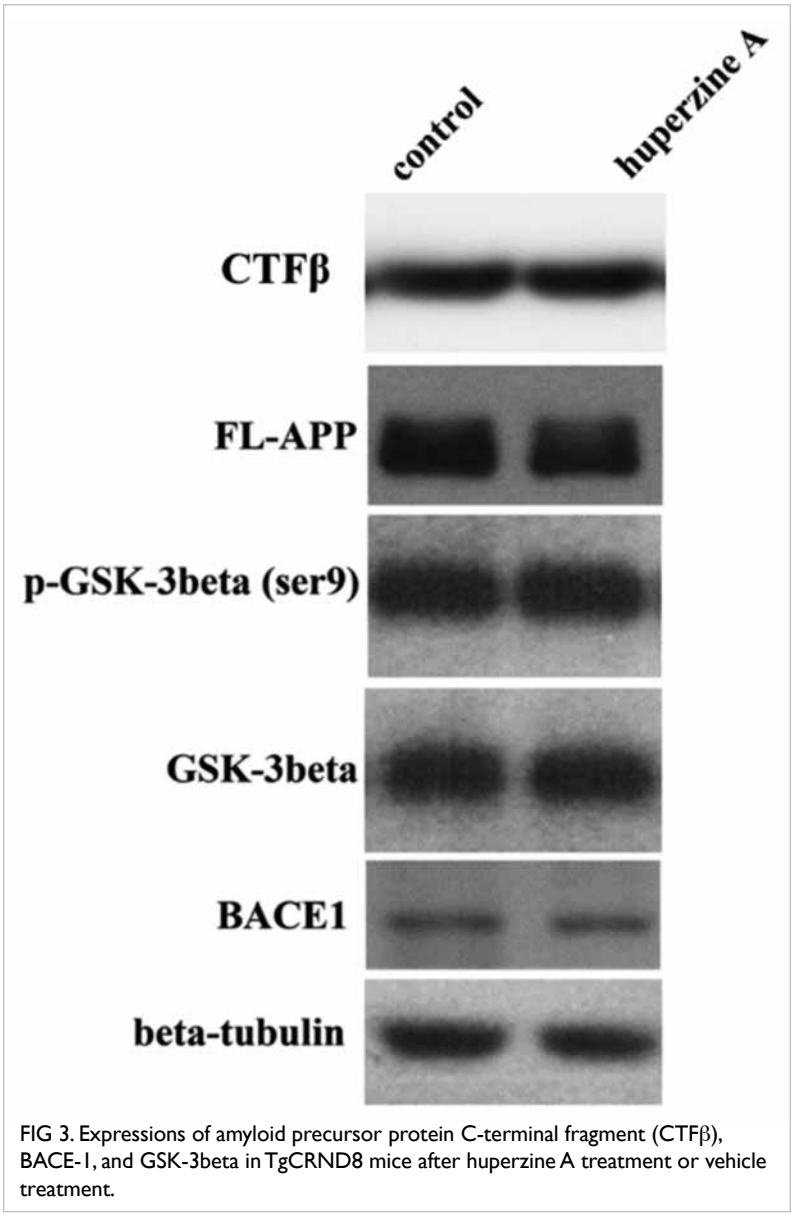


FIG 3. Expressions of amyloid precursor protein C-terminal fragment (CTFβ), BACE-1, and GSK-3beta in TgCRND8 mice after huperzine A treatment or vehicle treatment.

## Acknowledgements

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## Funding

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# Promoting physical activity among physically inactive people with impaired glucose tolerance and/or impaired fasting glucose: a clustered randomised controlled trial (abridged secondary publication)

**JTF Lau *, JCN Chan, SOL Pong, HHY Chung, R Ozaki, MCS Wong, EFL Leung, LWH Mui, KC Choi**

KEY MESSAGES

1. The intervention programme increased moderate/vigorous physical activity and related perceptions (eg perceived self-efficacy) among sedentary pre-diabetes patients.
2. Detection of pre-diabetic status plus supply of simple information improved moderate/vigorous physical activity levels.
3. Community-based screening could identify sedentary pre-diabetic cases.
4. Most participants were satisfied with the programme and would recommend it to others.

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## Introduction

The prevalence of diabetes mellitus (DM) among Hong Kong adults is high, as is the prevalence of pre-diabetes (impaired glucose tolerance and/or impaired fasting glucose). Up to 70% of pre-diabetes patients eventually develop DM.[1] Risk factors of DM include older age, obesity, family history of DM, physical inactivity, and gestational diabetes. The large-scale Da Qing randomised controlled trial reported that moderate/vigorous physical activity (MVPA) of >150 minutes/week reduced the DM incidence by 41.1% among patients with impaired glucose tolerance.[2]

The Health Belief Model was used to design the intervention programme. The model has been applied to promote physical activity, with perceived severity, perceived susceptibility, perceived benefits, perceived barriers, cue to action, and self-efficacy as determinants of health-related behaviours.[3] In Hong Kong, 47.7% of the population reside in public housing estates and are of lower socio-economic status and older age, which are factors of DM and low testing rate.

Using clustered randomised controlled trial design, we tested efficacy of a theory-based, support-group-based, and setting-based intervention in increasing MVPA level in the last week and related cognitions such as the Health Belief Model constructs among Chinese adults aged 40 to 69 years who had impaired glucose tolerance or impaired fasting glucose (based on the oral glucose tolerance test) and were sedentary (<150 minutes of MVPA in the last week). Mechanisms that potentially explain any intervention effect were explored.

## Methods

According to the 2006 World Health Organization, DM is defined as a fasting plasma glucose level of ≥7.0 mmol/L or a 2-hour plasma glucose level of ≥11.1 mmol/L. According to the American Diabetes Association, impaired glucose tolerance and impaired fasting glucose were defined as a fasting plasma glucose level of <7.0 mmol/L and ≥5.6 to ≤6.9 mmol/L, respectively, and a 2-hour plasma glucose level of ≥7.8 to <11.1 mmol/L and <7.8 mmol/L, respectively.

In phase I of the study, Chinese adults aged 40 to 69 years who resided in four public housing estates in Shatin were screened for DM risk using a risk-assessment questionnaire. Those who scored ≥12 and had <150 minutes of MVPA in the last week (based on the International Physical Activity Questionnaire [IPAQ]) were invited to take an oral glucose tolerance test.

In phase II of the study, inclusion criteria were (1) impaired glucose tolerance and/or impaired

fasting glucose, (2) <150 minutes of MVPA in the last week, and (3) provision of written informed consent. The four public housing estates in Shatin were equally randomised into the intervention and the control groups.

In the intervention group, there were three meetings to address different issues that participants might experience. Examples of meeting activities included presentations, games, contests, and exercise demonstrations, designed to generate participants' motivation to increase physical activity, reinforce their cognitive changes and foster their maintenance of MVPA level. After accredited instructors assessed participants personally to clear safety issues, participants were introduced a scheme to achieve MVPA of 150 minutes/week through easy-to-do activities, with personal adjustment suggested. Four support groups were formed. Group members performed weekly group brisk walking, joined subsidised social gathering, and encouraged each other to increase MVPA.

In the control group, participants received a booklet about physical activity only.

Outcome measures included the amount of MVPA (minutes) in the last week, oral glucose tolerance test result, blood pressure, and cognitions related to the Health Belief Model (e.g. perceived self-efficacy). Assessments were conducted at baseline and at month 7 and month 10 (1 and 4 months after completion of the 6-month phase II study).

## Results

Background characteristics of the intervention and control groups were comparable, including the prevalence of fulfilling the 150-minute MVPA recommendation. The intervention group was more efficacious in increasing the MVPA level than the control group. The intervention group also had significantly higher perceived self-efficacy, perceived susceptibility, perceived benefits, and perceived barrier than the control group.

Most participants in the intervention group found the programme useful/very useful in increasing knowledge on diabetes/benefits of physical activity, and received strong support from group members for increasing their MVPA. Over 90% of participants in both intervention and control groups felt satisfied/very satisfied with the programme and would recommend it to others. The intervention group showed greater subjective improvement in health and mood and stronger confidence in maintaining their physical activity level in the future than the control group.

At month 7, the intervention group showed significantly larger increase in perceived self-efficacy in performing the recommended MVPA level than the control group; perceived self-efficacy also partially explained the intervention effect on increasing mean MVPA time.

## Discussion

Residents of public housing estates were responsive to our screening plus free oral glucose tolerance test programme. This programme allows for earlier detection of DM. The control group that were given only simple printed materials showed significant increase in MVPA, with about 50% meeting the 150-minute MVPA recommendation at month 7 and month 10, compared with 0% at screening. Therefore, knowing the pre-diabetic status and being given basic health promotion significantly increased the MVPA level, with the increase sustained for a 10-month period. This finding justifies implementation of a large-scale community-based screening plus oral glucose tolerance test.

In the intervention group, the increase in MVPA level was even higher, with the prevalence of meeting the 150-minute MVPA recommendation being close to 70% at month 7 and month 10, compared with 0% at screening. It is likely that the higher level of MPVA achieved could be maintained, as both intervention and control groups showed high prevalence of behavioural intention to perform ≥150 minutes/week of MVPA at follow-up period.

Components corresponding to constructs of perceived self-efficacy, perceived barriers, perceived benefit, perceived susceptibility, perceived severity, and cue to action were built into the programme. The larger increase in perceived self-efficacy in the intervention group partially explained the intervention effect on the mean MVPA time. The findings were consistent with the theory used and the intervention contents.

The process evaluation further showed that not only was knowledge increased, participants subjectively felt improvement in health and mood, and were highly satisfied with the programme. Such positive experiences are important in maintaining newly formed health-related behaviours. Most participants would recommend the programme to others, confirming feasibility and acceptance of the programme.

The setting in public housing estates facilitated social interaction, group exercises, and utilisation of local resources (e.g. exercise classes held in the estates). Some participants in the intervention group pledged to serve as volunteers of similar programmes in the future.

### Limitations

The MVPA data were self-reported. The sample size was relatively small. The follow-up period was 10 months; longer-term intervention effect needs to be ascertained. The housing estates, though typical,

were not randomly selected, and included only four clusters. More studies are needed to understand responses of similar programme with and without subsidisation.

## Conclusion

The programme included (1) screening of sedentary individuals at-risk of DM using a risk assessment questionnaire, (2) identifying pre-diabetes using the oral glucose tolerance test, and (3) promoting MVPA among sedentary elderly people with pre-diabetes through a theory-based and support-group-based intervention. The intervention programme was effective in increasing MVPA levels and related cognitions. Perceived self-efficacy partially explained the intervention effect. Consistencies across the applied theory (Health Belief Model), the intervention design, and the findings demonstrated that this was a theory-based study. The study was menu-based and is highly replicable.

## Acknowledgements

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# Dedifferentiation-reprogrammed human mesenchymal stem cells for treating ischaemic stroke: abridged secondary publication

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## KEY MESSAGES

1. Dedifferentiated-reprogrammed human mesenchymal stem cells (De-neu-hMSCs) reveal distinguished stem cell phenotype such as enhanced neuronal differentiation potential, cell migration, and cell survival, compared with naïve hMSCs.
2. Systemic administration of hMSCs/De-neu-hMSCs significantly improves stroke recovery, with De-neu-hMSCs exhibiting stronger repair function.
3 De-neu-hMSC treatment results in more improved motor function recovery and less brain damage, compared with hMSC treatment.
4 The enhanced therapeutic effects of De-neu-hMSCs might be attributed to suppression of endogenous Bax-induced apoptosis pathway.

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## Introduction

Mesenchymal stem cell (MSC)–based therapy is a promising strategy in the treatment of stroke. However, low levels of MSC recruitment, cell survival, and directed differentiation in vivo largely limit their overall effectiveness and clinical use. Dedifferentiation reverts differentiated cells to an earlier, more primitive phenotype. Previous studies have demonstrated that dedifferentiation is a prerequisite for MSCs to change their cell fate and re-differentiate into a different lineage.[1] By manipulating cell fates of MSCs in vitro, we have found that after in vitro induction of neuronal differentiation and dedifferentiation, rat MSCs that have already committed to neuronal lineage revert to primitive cells distinct from naïve rat MSCs. The dedifferentiated rat MSCs exhibit enhanced cell survival and differentiation compared to unmanipulated rat MSCs in vivo, with significantly improved cognition function in a neonatal hypoxic-ischemic brain damage rat model.[2] These results indicate that dedifferentiated MSCs have potential to enhance the therapeutic effects in ischaemic brain disease. Thus, we hypothesise that dedifferentiation-reprogrammed human MSCs may exhibit enhanced therapeutic potential in treating stroke.

## Methods

We tested our hypothesis in both cell culture and rats with middle cerebral artery occlusion (MCAo) model. We aim to (1) characterise the dedifferentiation-reprogrammed human bone marrow-derived MSCs (De-neu-hMSCs), (2) compare the phenotypic properties (proliferation, cell survival, differentiation, migratory abilities) of De-neu-hMSCs with unmanipulated human MSCs in vitro, and (3) evaluate the therapeutic efficacy of De-neu-hMSCs and determine the mechanisms underlying the beneficial effects of De-neu-hMSCs in MCAo rat model.

## Results

After 24 hours of neural induction, >95% cells presented with neuron-like morphology (Fig. 1a). In line with the morphological changes, immunofluorescence analysis showed that the expression of neural markers Nestin and MAP2 were barely detectable in uncommitted hMSCs but significantly increased after 24 hours of neuronal induction (Fig. 1b). However, withdrawal of MNM rapidly reverted hMSC-derived neuron-like cells to characteristic mesenchymal morphology, and suppressed Nestin and MAP2 expression within 24 hours (Fig. 1b). These De-neu-hMSCs from differentiated neuronal cultures could be re-induced into neuronal phenotype upon re-exposure in MNM without pre-induction with ATRA and bFGF. We then further characterised immunophenotypic

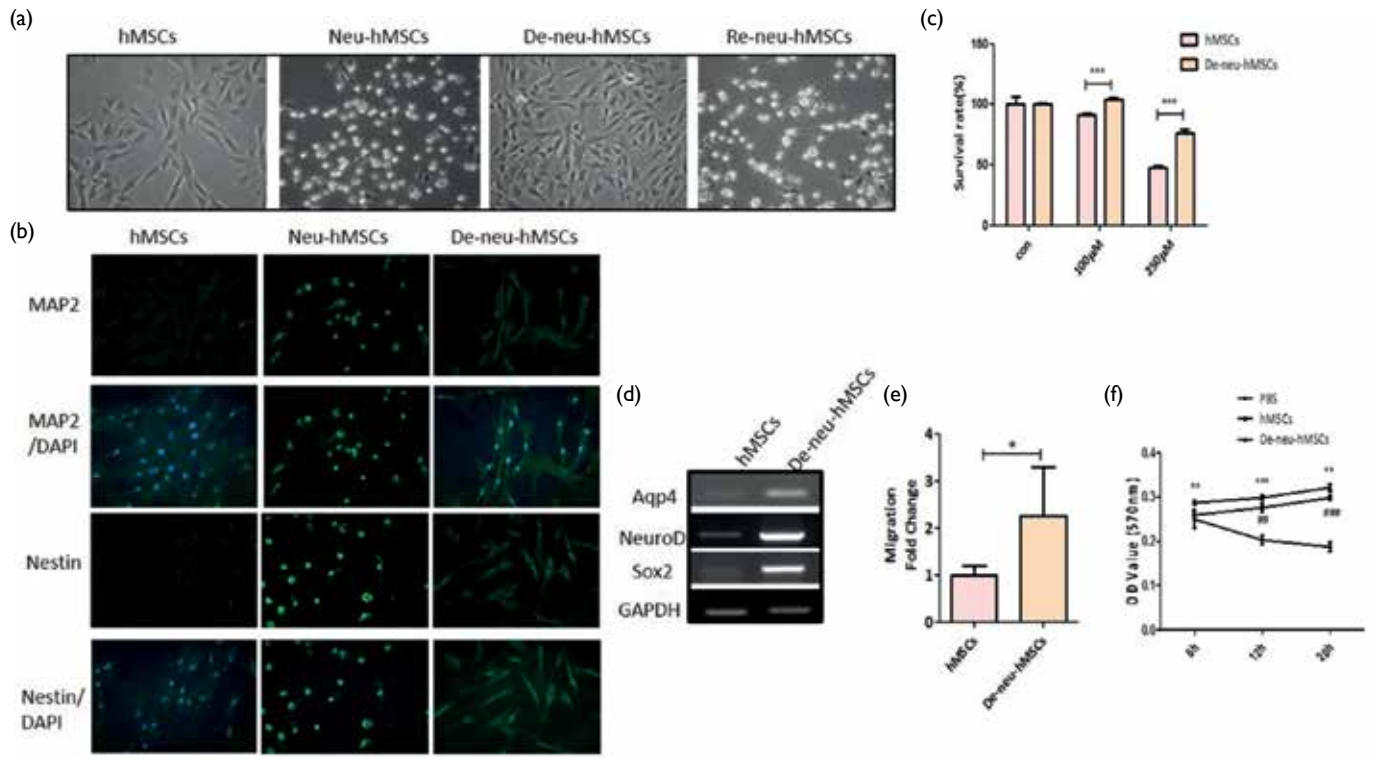


**FIG 1. Neuronal differentiation, dedifferentiation, and re-differentiation of human bone marrow mesenchymal stem cells (hMSCs).** (a) Phase contrast photographs of neuronal differentiation, dedifferentiation, and re-differentiation of hMSCs. (b) Immunostaining of the neuronal markers: MAP-2 and Nestin. (c) Dedifferentiation-reprogrammed hMSCs (De-neu-hMSCs) exhibit survival advantage over untreated hMSCs. The untreated hMSCs and De-neu-hMSCs are plated in 96-well plates and challenged with 0-250 μM $H_2O_2$ for 24 hours. (d) RT-PCR analysis of Aqp4, Sox-2, and NeuroD in hMSCs and De-neu-hMSCs. (e) De-neu-hMSCs exhibit advantage in migration over untreated hMSCs. (f) The condition media derived from either hMSCs or De-neu-hMSCs are added into serum-deprived and low glucose treated PC-12 cells for 24 hours. Cell proliferation is assessed using MTT assay. The absorbance at 450 nm is measured.

marker in hMSCs/De-neu-hMSCs. Fluorescence-activated cell sorting showed that De-neu-hMSCs retained their immunophenotype similar to that of undifferentiated hMSCs. These results indicate that dedifferentiation can be achieved in human MSCs.

We then used various cell functional analyses to characterise the De-neu-hMSCs. De-neu-hMSCs exhibited a survival advantage over undifferentiated hMSCs when challenged with hydrogen peroxide (Fig. 1c). In addition, the expression level of neural markers decreased upon dedifferentiation, with the level higher in De-neu-hMSCs than in undifferentiated hMSCs (Fig. 1b). RT-PCR analysis revealed that the expression of neural progenitor markers such as NeuroD1, Sox-2, and Aquaporin 4 was much higher in De-neu-hMSCs than in hMSCs (Fig. 1d). These results are consistent with our previous study that De-neu-rMSCs have enhanced cell survival and higher potential to re-differentiate into neurons.[2]

We then proceeded to evaluate the effect of dedifferentiation on the migratory ability of hMSCs. De-neu-hMSCs exhibited enhanced migratory ability as demonstrated by transwell assay (Fig. 1e). Oxygen glucose–deprived PC-12 cells were co-cultured with hMSCs or De-neu-hMSCs for 24 hours. Co-culture dramatically increased the number of viable cells after oxygen and glucose deprivation, with a significantly larger number of cells observed with De-neu-hMSCs than with hMSCs (Fig. 1f), indicating enhanced cell survival. Collectively, these results indicate that the enhanced stemness observed in the De-neu-MSCs represents a general property among MSCs derived from different species.

The therapeutic efficacy of De-hMSCs was evaluated using the middle cerebral artery occlusion (MCAo) rat model. A total of 140 adult Sprague Dawley rats were used. In the preliminary experiment, 20 rats were used to establish focal cerebral ischaemia and 15 rats were used to establish intracarotid stem cell delivery. In the first batch of experiments, 45 rats were divided into PBS, hMSC, and De-neu-hMSC groups (15 per group). At day 1 and day 10 after stem cell transplantation, the severity of MCAo-induced motor and sensory deficits were assessed by the modified Neurologic

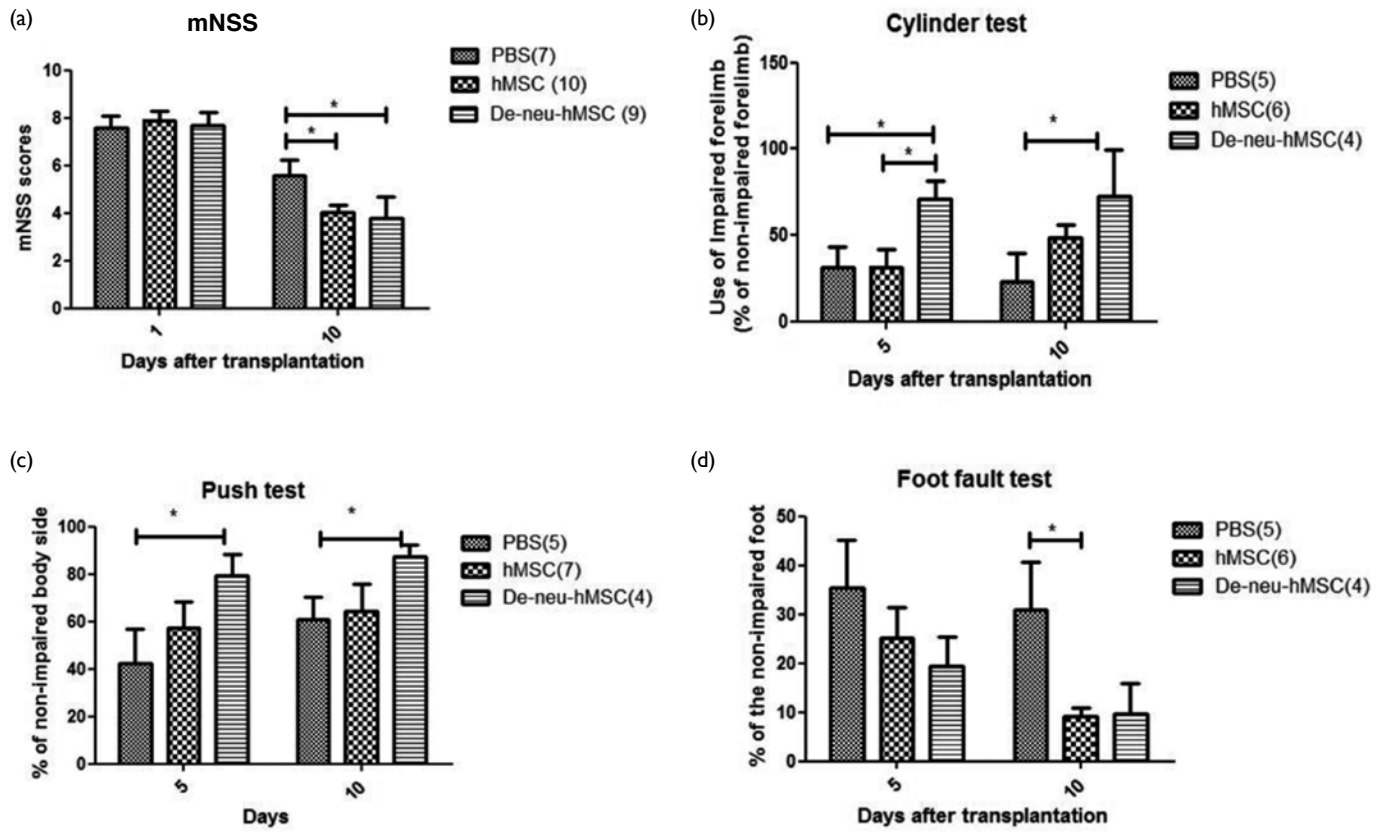


**FIG 2. Dedifferentiation-reprogrammed human bone marrow-derived mesenchymal stem cells (De-neu-hMSCs) significantly improves motor function recovery in rats with middle cerebral artery occlusion.** (a) hMSC and De-neu-hMSC transplantation improve modified Neurological Severity Score (mNSS). (b) De-neu-hMSC transplantation improves cylinder test performance at days 5 and 10 after transplantation. (c) De-neu-hMSC transplantation improves push test performance. (d) hMSC and De-neu-hMSC transplantation improve foot fault test performance at day 10 after transplantation.

Severity Score (mNSS). In the second batch of experiments, 30 rats were used for behaviour tests (cylinder test, push test, and foot fault test) at day 5 and day 10 after stem cell transplantation. In the third batch of experiments, 30 rats were randomly assigned to three groups. At day 7 after stem cell transplantation, rats were deeply anaesthetised and the brain was fixed by trans-cardiac perfusion of 4% PFA. The fixed brain was embedded in optimal cutting temperature, and 5-μm coronal sections were cut by cryostat.

Neurological function of rats was assessed using the mNSS at days 1 and 10 after stem cell transplantation. The mNSS is a composite test for motor (muscle status, abnormal movement), sensory (visual, tactile, and proprioceptive), and reflex,[3] with scores ranging from 0 (normal) to 18 (maximal deficit). The mNSS was close to 8 in MCAo rats on day 1 post stem cell treatment, indicating neurological functional deficits. Significant reduction in the mNSS was found in the PBS-treated animals compared with day 1 after injury (Fig. 2a), suggesting that a significant spontaneous sensorimotor functional recovery occurred after MCAo. Functional recovery was significantly higher in the hMSCs and De-neu-hMSCs groups than in the PBS group. There was no significant difference between hMSCs and De-neu-hMSCs groups at day 10 (Fig. 2a).

For the cylinder test, at day 5 after stem cell treatment, only De-neu-hMSCs–treated animals exhibited decreased forelimb asymmetry. At day 10 both hMSCs and De-neu-hMSCs groups exhibited decreased forelimb asymmetry, but this effect was significant only in rats receiving De-neu-hMSCs, compared with control rats receiving PBS alone (Fig. 2b).

For push test, De-neu-hMSCs treatment produced a significant increase in percentage resistance to lateral push after day 5 and day 10, when compared with the PBS treatment. However, the hMSC group did not have this effect (Fig. 2c).

The foot fault rate of the affected limb is the ratio of the number of fault footsteps to the total number of steps. The test was repeated three times and was performed on day 5 and day 10 after stem cell treatment. At day 5, the foot fault rate in the

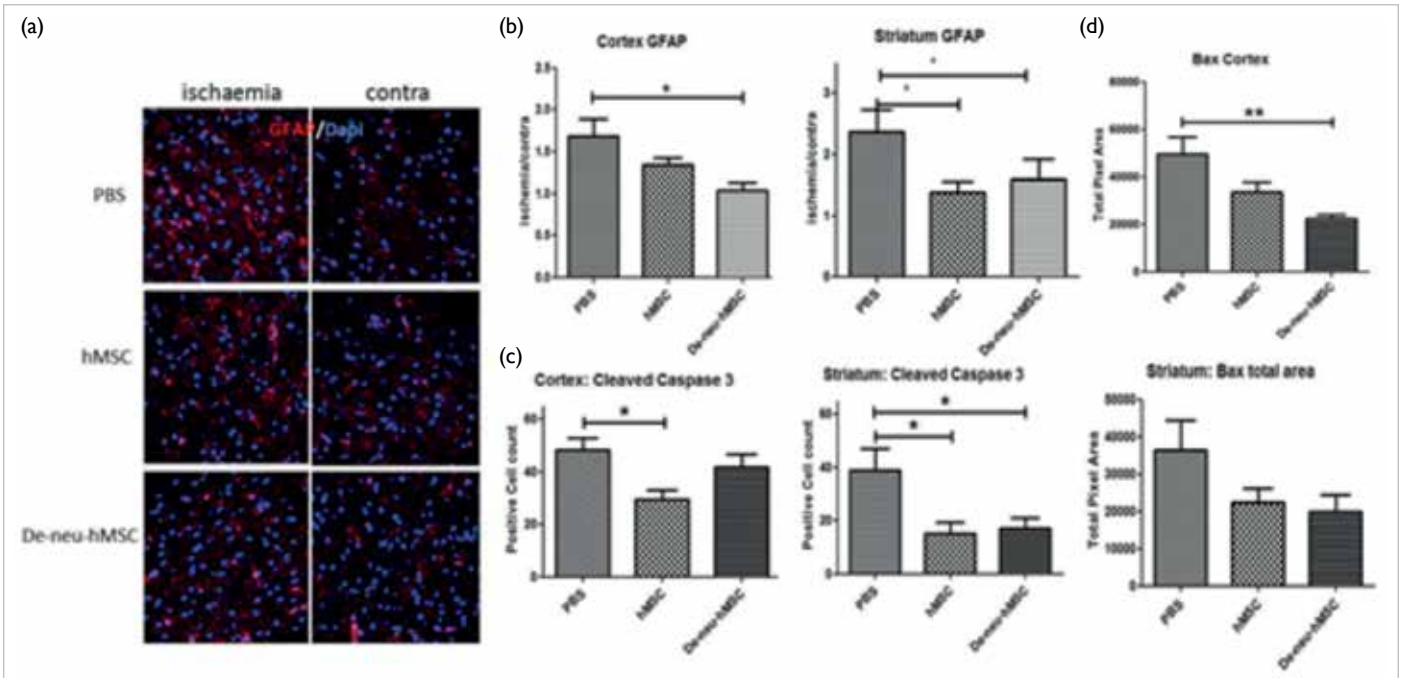


**FIG 3. Dedifferentiation-reprogrammed human bone marrow-derived mesenchymal stem cells (De-neu-hMSCs) alleviates brain damage and decreases Bax expression in rats with middle cerebral artery occlusion.** (a) hMSC and De-neu-hMSC transplantation reduce GFAP$^+$ cells in the cortex and striatum. (b) GFAP$^+$ cells are calculated in both ischaemia and its contra areas and the ratio of ischaemia/contra are used to evaluate the increased GFAP positive cells in both cortex and striatum areas. (c) Cleaved Caspase 3 positive is significantly decreased in hMSC or De-neu-hMSC groups. (d) Bax expression intensity is significantly decreased in the De-neu-hMSC group.

hMSC and De-neu-hMSC groups were mildly lower than that in the PBS group. By contrast, at day 10, the foot fault rate in the hMSC group and De-neu-hMSC group was significantly lower than that in the PBS group, while there was no significant difference between hMSCs and De-neu-hMSCs groups (Fig. 2d).

To further determine the molecular mechanism underlying the therapeutic effects of MSCs/De-neu-hMSCs, rats were deeply anaesthetised and the brains were fixed by trans-cardiac perfusion of 4% PFA for immunofluorescent staining. GFAP-staining was used to identify reactive astrocytes in the brain after MCAo. When neurons are damaged by ischaemia, astrocytes will generate to fill the space of dead neurons. Thus, the expression level of GFAP indicates the degree of brain damage. MCAo alone significantly increased the percentage of GFAP$^+$ cells in the lesion boundary zone of the injured hemisphere compared to the contra-side. hMSC/De-neu-hMSC treatment significantly reduced the GFAP$^+$ astrocyte in the injured striatum compared to the PBS treatment. De-neu-hMSCs (but not hMSCs) significantly reduced the GFAP$^+$ astrocyte in the injured cortex (Fig. 3). Our results suggest that stem cell treatment alleviates brain damage, more prominent in the De-neu-hMSC group. We then stained the brain tissue with cleaved caspase-3 to evaluate the apoptotic response in untreated or stem cell–treated MCAo models. Both hMSC and De-neu-hMSC treatment significantly decreased cleaved caspase-3 positive cells compared to PBS treatment, indicating stem cell treatment alleviates apoptosis-induced damage in MCAo model (Fig. 3c). We then determined the expression intensity and positivity of Bax and Bcl-2, which are apoptosis regulators. Stem cell treatment significantly decreased expression intensity and positivity of Bax (but not Bcl-2). The suppressive effect on Bax expression was more prominent in De-neu-hMSC group (Fig. 3d).

## Conclusion

Human MSCs can be reprogrammed via in vitro neuronal differentiation and dedifferentiation, with enhanced cell survival, neural differentiation capacity, and migration. De-neu-MSCs exhibit enhanced therapeutic effects in treating stroke, probably through suppression of Bax-induced apoptosis pathway. Further studies are warranted to identify the molecular mechanism underlying the enhanced therapeutic effects of De-neu-hMSCs. With easy culture manipulation and low tendency of tumour formation, dedifferentiation strategy provides a feasible approach to enhance therapeutic efficacy for

stroke and stem cell based-regenerative medicine. The proposed research work has strong clinical relevance and great potential for clinical applications.

## Funding

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## Disclosure

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1. Yang FY, Zhang XH, Tsang LL, Chan HC, Jiang XH. Dedifferentiation-reprogrammed mesenchymal stem cells for neonates with hypoxic-ischaemic brain injury. Hong Kong Med J 2019;25(Suppl 5):12-6.

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# Dysregulation of miR223 and miR431 expression in intestinal tissues of preterm infants with necrotising enterocolitis: abridged secondary publication

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KEY MESSAGES

1. The miR-223/*NFIA* and miR-431/*FOXA1* pathways were aberrantly expressed in intestinal tissues of patients with necrotising enterocolitis.
2. The affected downstream signals could dysregulate multiple categories of cellular functions and play important roles in disease pathophysiology.

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## Introduction

Necrotising enterocolitis (NEC) is a severe inflammatory disease of the gastrointestinal tract that results in high morbidity and mortality in preterm infants. Micro-RNAs (miRNAs) are a class of noncoding small RNAs (18-24 nucleotides) that exert post-transcriptional inhibition of gene expression by pairing with complementary sequences in target mRNAs. Specific miRNAs play important roles in physiologic events, and their dysregulation is associated with diverse pathologic conditions. miRNAs have been implicated in ileal and colonic mucosal as well as blood specimens of patients with Crohn disease and ulcerative colitis. This suggests that miRNAs can interfere with gut inflammation.[1-3] We reported the mRNA and miRNA expression profiles of NEC intestinal tissues, and miR-223 and miR-431 were significantly upregulated when compared with those in surgical control tissues (ie non-inflammatory neonatal surgical conditions).[4] The objectives of the current study were: (1) to validate the dysregulation of miR-223 and miR-431 in NEC tissues, (2) to identify the direct binding target genes of these two miRNAs, and (3) to investigate their functions associated with NEC pathophysiology.

## Methods

Intestinal specimens were collected during surgery of infants with stage III NEC at the Prince of Wales Hospital, The Chinese University of Hong Kong, as described previously.[4] Written informed parental consents and institutional ethics approval were obtained. Surgical control tissues were obtained from infants who underwent intestinal surgery because of non-inflammatory conditions. Target genes of miR-223 or miR-431 were identified by in silico target prediction bioinformatics, luciferase assay, and Western blot analyses. Expression levels of miR-223, miR-431, and downstream regulatory genes were measured by qPCR. Functions of miR-223 and miR-431, including expressions of target genes and downstream signals, cell proliferation, and apoptosis were investigated by overexpression in Caco-2 and/or FHs74 cells upon stimulation by lipopolysaccharide or lipoteichoic acid. Regulatory networks were analysed by Metacore Analysis.

Expression levels of regulatory genes in NEC and surgical control tissues were compared using the unpaired *t* test. Correlation analyses between miRNAs and regulatory genes were performed using the Spearman correlation test. Data of luciferase reporter assay, Western blot, and cell apoptosis assays were analysed by the paired t test. The proliferation of mimic-miR or mimic control–transfected cell lines were compared using two-way ANOVA with Bonferroni correction. Statistical analyses were performed with the GraphPad Prism 5.0 software.

## Results

The expression of miR-223 was significantly upregulated in NEC tissues by 25.16-fold, compared with surgical control tissues. Nuclear factor I-A (*NFIA*) was identified as the target gene of miR-223. Overexpression of miR-223 significantly regulated multiple downstream genes, including *MYOM1*, *NFIA*, *RGN*, *GNA11*, *MYLK*, *PRKCZ*, *IL-6*, and *IL-8* in Caco-2 and FHs74 cells. In addition, apoptosis

was significantly increased and proliferation was inhibited. These results suggested that upon binding with *NFIA*, miR-223 could regulate functional effectors in pathways of apoptosis, cell proliferation, G-protein signalling, inflammation, and smooth muscle contraction.

The expression of miR-431 was significantly increased by 7.05-fold in NEC tissues, compared with surgical control tissues. Forkhead box A1 (*FOXA1*) was validated as a target gene of miR-431. *IL6*, *IL8*, and *TNF* were significantly increased upon overexpression of miR-431 and lipopolysaccharide or lipoteichoic acid treatments. In addition, *HNF4A* was decreased, whereas *NFKB2* was increased. Functional analyses demonstrated that cell proliferation was significantly decreased and apoptosis was increased upon overexpression of miR-431. Overall results indicated that the miR-431/*FOXA1* axis was proinflammatory and could be associated with NEC pathology.

## Discussion

Our study presented the first evidence that the miR-223/*NFIA* and miR-431/*FOXA1* pathways were dysregulated in intestinal tissues of NEC infants, and that affected downstream signals could probably reprogram the expression of effector genes in multiple categories of cellular functions. We also observed increased apoptosis and decreased proliferation of intestinal cells overexpressing miR-233 or miR-431, and/or upon exposure to bacterial toxins. Our findings strongly indicated that dysregulation of miR-223/*NFIA* and miR-431/*FOXA1* as well as downstream regulatory genes could contribute to the pathophysiology of NEC, impacted by escalated inflammation, disturbed homeostasis, increased apoptosis, and suppressed cell proliferation, and thus leading to irreparable tissue damage. Our findings provided new insights into the molecular mechanism of NEC upon regulation by miRNAs and shed lights on potential therapeutic targets for these vulnerable infants.

## Funding

This study was supported by the Health and Medical Research Fund, Food and Health Bureau, Hong Kong SAR Government (#02130566). The full report is available from the Health and Medical Research Fund website (https://rfs1.fhb.gov.hk/index.html).

## Disclosure

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