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Immunotherapy for Nasopharyngeal Carcinoma: Targeting the Epstein-Barr Virus

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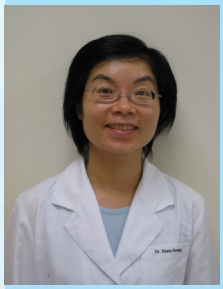

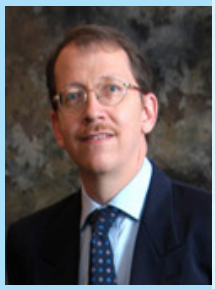

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國際市場准入：亞洲新興市場概述

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A Word from the Editor in Chief

Prof. Jack Wong,
Editor in Chief
Director, Regulatory Affairs, Asia Pacific,
Terumo BCT (Asia Pacific) Ltd.
(Singapore Branch)
Email: speedxquality@yahoo.com

Dear Readers,

Thank you for reading Asia Health Care Journal issue January 2016. We are honored to have the publication of the sixth edition. Several healthcare experts, who are in different specialties such as Ophthalmology and Biomedical Sciences, have contributed their researches. In the meantime, students from the Chinese University of Hong Kong and the Hong Kong University of Science and Technology are actively participating in this journal.

In this year, regulatory training of our Association has been expanded in Japan (Tohoku University) and China (Peking University) respectively. Through this platform, we hope to promote the experience-sharing among experts in different fields and foster the development of the healthcare standard in Asia.

Finally, I would like to once again pay tribute to our team and hope that you could enjoy reading this journal.

Wish you and your love ones happy and healthy in 2016!

Prof. Jack Wong
Asia Regulatory Professional Association

ARPA
Asia Regulatory Professional Association

The Asia Regulatory Professional Association (ARPA) is an organization of healthcare regulatory affairs professionals in Asia. ARPA aims to raise the standard and social recognition of regulatory professionals as part of healthcare team.

Details of ARPA can be found in
http://www.healthcare.org.hk/Content.aspx?t1=22&t2=79

Values of Asia Regulatory Professional Association (ARPA)

To uphold and enhance standards among regulatory affairs professionals in Asia and to encourage the creation of better educated regulatory teams in the area, regardless of the background and regulatory situation of their countries. A new body, the Asia Regulatory Professional Association (ARPA), was established in 2010 with more than 3,500 members today.

Structure

ARPA strives to be neutral. There is a good balance of key individuals from different countries as well as from academic and regulatory bodies.
The ARPA chairman is Dr. Saleh S. Al-Tayyar from Saudi FDA and co-chairman is Madam Liu Li-Ling from Taiwan FDA. Dr. Saleh and Madam Liu are also the chairman and co-chairman in Asia Harmonisation Working Party (AHWP) to help avoiding duplication with relevant work that is ongoing within that organization which aims to work towards greater harmonization in medical device regulations in Asia.
Prof. Rosanna Peeling is our advisor (ex-WHO staff, now working in London University).

HKU-QIMR BERGHOFER cooperate to help Nasopharyngeal Carcinoma patients

Left to Right: Prof. John Nicholls, Ms. Angela To, Prof. Frank Gannon, His Excellency the Honorable Paul de Jersey AC, Prof. Gabriel M. Leung, Mr. Paul Tighe, Prof. Rajiv Khanna

The Li Ka Shing Faculty of Medicine of the University of Hong Kong and QIMR Berghofer Medical Research Institute (QIMR Berghofer) in Australia have taken a great step to collaborate to help Nasopharyngeal Carcinoma (NPC) patients to access a ground-breaking cancer treatment.

Representatives of the two institutions have signed a memorandum of understanding (MOU) on 28th Aug, 2015 in Hong Kong with the attendance of His Excellency the Governor of Queensland, the Honorable Paul de Jersey AC. The MOU also initiates a training programme for Hong Kong clinical or science graduates to master scientific techniques of producing clinical grade killer T-cell for immunotherapy.

Scientists from the two institutions work together for the second phase trial using immunotherapy for the management of NPC. Professor Khanna from QIMR Berghofer's Tumour Immunology Laboratory said that the Phase I trial, which started in 2009, involved late-stage NPC patients. The Phase I trial not only confirmed the safety of the immunotherapy but also improved patients' life expectancy. Phase II trial has started in September 2015. Thirty newly diagnosed patients with metastatic NPC at the Queen Mary Hospital in Hong Kong and others in Australia are involved in this trial.

About QIMR Berghofer

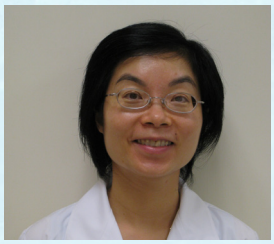
QIMR Berghofer Medical Research Institute

QIMR Berghofer is a world leading translational research institute focused on cancer, infectious diseases, mental health and a range of complex disorders. Working in close collaboration with clinicians and other research institutes, their aim is to improve health by developing new diagnostics, better treatments and prevention strategies.

Prof. Gabriel M. Leung, Dean of the Li Ka Shing Medical Faculty, HKU, describes the collaboration as a small beginning to something potentially very big and very impactful

Prof. Frank Gannon, Director and CEO of QIMR Berghofer speaks on its work and the importance of the collaboration with HKU

His Excellency, Hon. Paul de Jersey AC, calls for the strengthening of relationship between Queensland and Hong Kong



Prof. Dora Lai Wan Kwong

Prof. Dora Kwong is currently a Clinical Professor in Department of Clinical Oncology, the University of Hong Kong. Her research interests include head and neck cancer with an emphasis on nasopharyngeal carcinoma (NPC), upper GI cancers including CA esophagus and CA stomach and paediatric cancers. She is involved in multiciplinary management employing both medical treatment and radiotherapy. She is also interested in basic research in carcinogenesis and translational research in identifying prognostic and predictor biomarkers. Prof. Kwong is a recognised expert in her field of research and has been invited to speak in international meetings and author book chapters.

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Prof. John Nicholls

Prof. John Nicholls is a Clinical Professor in Pathology at the University of Hong Kong. He commenced medical studies at the University of Adelaide, South Australia in 1977 and graduated in 1983. He commenced postgraduate training in pathology at the Institute of Medical and Veterinary Sciences, the Queen Elizabeth Hospital and The Adelaide Children's Hospital. In 1988 he moved to Hong Kong as a Lecturer in Pathology at the University of Hong Kong where in addition to clinical and teaching duties commenced research into the relationship of viruses with the respiratory tract. His current investigative work is looking at the viral binding sites in the respiratory tract and determining susceptibility to avian influenza in humans and other animals. Together with staff from the School of Public Health he has established a lung and bronchial ex vivo culture system to investigate tropism and pathogenesis of emerging viral infections, as well as potential novel antiviral agents such as DAS181 in these systems. In 2009 he was awarded a Croucher Senior Medical Fellowship to work on novel therapeutic strategies for influenza.

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Dr. Victor Lee is currently a Clinical Assistant Professor of the Department of Clinical Oncology, the University of Hong Kong. He graduated the University of Hong Kong in 2002. He continued his studies in clinical oncology and received specialist training in interstitial brachytherapy for head and neck cancers and sarcoma, stereotactic body radiation therapy for liver tumors, etc. In 2015 he was awarded HKCR 15A Traveling Fellowship and pursued subspecialty training in image-guided brachytherapy for cervical cancer and pediatric oncology.

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Immunotherapy for Nasopharyngeal Carcinoma: Targeting the Epstein-Barr Virus

Abstract

Nasopharyngeal carcinoma is associated with type II latent EBV infection and tumor cells expressed viral antigens which are attractive targets for treatment. Tumor cells survival is dependent on their ability to evade T-cell immunity. Immunotherapy targeting the EBV antigens by restoring T cell population and function is a promise strategy. In this article, we shall review the different immunotherapy approaches and the results from preliminary clinical studies. With the recent interest in non-target specific immunotherapy using immune checkpoint inhibitors, the potential of immunotherapy using combination approach will be worth further study.

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is endemic in Southern China and Southeast Asia with an annual incidence of 15-24 cases per 100,000 people. It is the most common head and neck cancer in Hong Kong and ranked 8th in incidence and 9th in mortality in 2012. Although the cure rate of NPC has much improved with the advance in chemotherapy and radiotherapy, still around one third of patients would relapse after primary treatment, mostly with distant metastases which are uniformly fatal. Relapsed cases in modern era are even more difficult to treat than in the past since most of them would have been exposed to intensive chemoradiotherapy before which makes these tumor likely to be resistant to chemotherapy and radiotherapy and also at risk of increased toxicity from repeated exposure to these conventional treatment. There is a rapid expansion in our armaments in treatment of cancers in the last decade due to the development of many target therapeutic agents. However, so far there is no proven effective target therapy for metastatic NPC and there is real need in the search for novel treatment especially for relapsed, metastatic cases. Most endemic NPCs are poorly or undifferentiated carcinomas and are almost always associated with Epstein-Barr virus (EBV). The viral antigens expressed by the tumor cells are attractive target for immunotherapy. However, the host immune surveillance failed to detect these viral antigens in NPC because there are only limited viral antigens expressed which are not immunogenic. Also, previous studies based on murine models showed that high viral load can induce T-cell exhaustion and / or anergy[1]. Thus, we need to find ways to restore the host immunity against this EBV-related malignancy. That can be achieved by passive transfer of T lymphocytes targeting the EBV or by active immunization against EBV. To understand the rationale in targeting EBV in immunotherapy, we have to understand the role of EBV latent infection in carcinogenesis.

EBV latent infection[2]

The EBV infection is ubiquitous in adult humans. EBV primarily infects the human oropharynx epithelial cells and then replicates and spreads to B cells, resulting in latent infection in B cells, epithelial cells and natural killer/T cells (NK-T cells) after extensive host T-cell immune surveillance.

Latent EBV plays a role in carcinogensis of different cancers including Burkitt's lymphoma, Hodgkin's lymphoma, lymphoproliferative disease, NK-T cell lymphoma and endemic NPC. There is persistence of EBV genomes in all cells of these malignancies, consistent with the notion that EBV latent genes are important for malignant cell growth.

Latent EBV genomes express five EBV-encoded nuclear antigens (EBNA-1, -2, -3A, -3B,-3C) and two latent membrane proteins (LMP-1, -2A), EBV-encoded small RNA (EBER) and non-transcribed BART (BamHI-A region rightward transcript) RNAs. There are three distinct latent EBV infection statuses: latency types I, II and III depending on the viral gene expression pattern. Type I latency is associated with Burkitt's lymphoma. Type II latency is associated with Hodgkin's lymphoma and NPC. Type III latency is found in actively proliferating (post-transplantation) lymphoproliferative disease.

NPC display type II latency where only a limited array of EBV antigens, including latent membrane proteins (LMP) 1 and 2 and EBV nuclear antigen 1 (EBNA1) are expressed. LMP-1 and LMP-2 expression likely contributes to cell survival by activation of nuclear factor-kB (NF-kB) and phosphatidy inositol 3 kinase (PI3K) pathways. EBNA-1 peptides are poorly presented in the context of major histocompatibility complex class I. Cells expressing EBNA-1 are therefore partially protected from recognition by CD8 cytotoxic T lymphocytes.

Post-transplant lymphoproliferative disease (PTLD) and in vitro EBV infection-mediated establishment of the lymphoblastoid cell line (LCL) both show type III latency in which most latent genes are expressed (EBER1/2 RNA, EBNA-leader protein (EBNA-LP), EBNA-2, EBNA-3 ABC, EBNA-1, LMP-2A/B, LMP-1 protein, BART RNA). PTLD occurs in lymphopenic immunocompromised bone marrow transplant and solid organ transplant patients. Adoptive immune transfer with reactivation and expansion of T cells ex vivo using LCL from healthy seropositive donors effectively treat EBV associated PTLD[3][4]. The ability of these antigen specific T cells to discriminate between normal and tumor tissues and corresponding limited toxicities simulate similar approaches to be studied in other EBV-associated malignancy including NPC.

Adoptive T-cell transfer for NPC

In 2001, our group reported our pilot study in use of adoptive transfer of autologous EBV-specific cytotoxic T cells for NPC[5]. We first determined the EBV-specific CTL precursors in peripheral blood from 22 NPC patients, 4 disease-free survivors and 7 healthy EBV carriers. There was evidence that quantitative immune deficit in CTL precursor level was related to burden of disease. The plasma EBV DNA load increased when the CTL precursor level fell below 150/10[6]. CTL levels were lower in NPC patients compared with control and correlated with progression of disease. CTL precursor level in survivors was restored to levels similar to those in healthy carrier after successful treatment. Based on this findings, 4 patients with advanced NPC were treated with an infusion of EBV CTLs. EBV CTLs were expanded ex vivo by repeatedly stimulating peripheral blood monocytes with irradiated EBV-transformed B-lymphoblastoid cell line (LCL) established from the same patients and culturing the cells in the presence of IL-2. We found that the treatment is safe and restored host surveillance of EBV replication, reducing the plasma EBV burden, although no objective response was observed. Subsequent studies on the use of LCL induced autologous EBV specific CTL for NPC had been reported[6][7][8]. Louis et al. reported 5 out of 15 patients treated with active disease achieved complete remission[9].

Traditionally, EBV-CTLs have been generated by weekly stimulation of donor PBMCs with autologous EBV-LCLs in the presence of IL-2. This is a prolonged process, requiring 4-6 weeks for EBV-LCL establishment with a further 4 weeks to generate sufficient CTLs for both testing and infusion. Also, the EBV-specific CTL generated by stimulation with EBV-LCL favored the outgrowth of CTL responses to the immunodominant EBNA-3-6 antigens rather than the subdominant EBV LMP-1 and LMP-2 expressed in NPC. Methods that provide for rapid T-cell expansion and increasing the specificities of CTL for the EBV latency II antigens expressed in NPC would increase efficiency in treatment and enhance anti-tumor response.

In collaboration with researchers from Queensland Institute of Medical Research (QIMR) in Australia, we performed another Phase I study using a novel adenovirus-based adoptive immunotherapy for NPC[10]. In this study, an adenovirus-based vector, referred to as AdE1-LMPpoly, which encodes multiple CTL epitopes from LMP1 and 2 fused to a truncated EBNA1 without an internal glycine-alanine repeat sequence, was used to rapidly expand LMP1 and 2 and EBNA1-specific T cells from patients. Patients with recurrent and metastatic NPC who have failed other treatments were recruited in Hong Kong. Blood was drawn from patient and sent to Australia for generation of LMP/EBNA1-specific T cells in accordance with Good Manufacturing Practice in the Q-Gen facility in QIMR. The harvested and cryopreserved CTLs were sent back to Hong Kong for infusion back to patients. The preliminary results were reported in 2012 in Cancer Research. A total of 22 patients were analyzed, 17 patients with distant metastases and 5 patients with advanced loco-regional recurrence who have failed multiple lines of palliative chemotherapy. The AdE1-LMPpoly vector was found to be highly efficient in expanding antigen-specific T cells. The expanded T cells display high levels of functional capacity as assessed by IFN- expression. Only 2 weeks are required for T cell expansion and the T cells are specific for LMP-1&2 and EBNA1. CTL was successfully expanded in 16 patients. Six patients failed T cell expansion, probably due to their very low white cell counts. Fourteen patients had CTL treatment. The treatment was safe with only grade 1 flu-like symptoms and malaise observed. Of the 14 patients treated with CTL, 10 patients showed stable disease and the time to progression ranged from 38 to 420 days, with a mean time to progression of 136 days. Although no objective response was observed after CTL infusion, one patient showed dramatic shrinkage of tumor on receiving chemotherapy after CTL infusion. Transient increase in the frequencies of LMP1&2 and EBNA1-specific T cell responses was observed after adoptive transfer. We concluded that adoptive immunotherapy with AdE1-LMPpoly-stimulated T cells is safe and may provide long-term clinical benefit is stabilization of relapsed NPC. We have recruited additional patients after this preliminary analysis. The Phase I study is now closed and pending further data analysis. In the further, we plan to further test this strategy in adoptive immunotherapy for recurrent or metastatic NPC in the first line setting in combination with chemotherapy. At present, in Hong Kong, we do not have GMP qualified facilities for virus-specific T cells expansion, thus patients' blood need to be sent overseas for processing. We hope this technology can be transferred to Hong Kong in the near further so that the treatment can be more cost-effective, efficient and can be expanded to benefit other patients with viral-related malignancies.

Therapeutic vaccine against EBV

Besides adoptive immune transfer, another approach will be by inducing cellular immunity in vivo by therapeutic vaccines. Two types of therapeutic vaccines have been tried for patients with nasopharyngeal carcinoma. Taylor et al. expressed a fusion protein containing the carboxyl terminus of EBNA-1 fused to LMP2 in a poxvirus vector (modified vaccinia Anakara) [11]. They showed that dendritic cells infected with the recombinant poxvirus reactivated LMP2-specific CD8 T cells and EBNA-1 specific memory T cells in vitro using peripheral blood mononuclear cells from healthy seropositive persons. This vaccine has been tested clinically. Hui et al. vaccinated NPC patients in remission with the vaccine and detected increased T-cell responses to at least one viral protein in 15 of 18 patients and a three to fourfold increase in the magnitude of T-cell responses to the proteins[12]. A follow-up study in the United Kingdom with the same vaccine showed that 8 of the 14 patients tested had increased CD4 and CD8 T-cell responses after vaccination to one or both EBV proteins[13].

In a second approach, autologous dendritic cells were incubated with EBV peptides or infected with viral vectors expressing EBV proteins and then injected into patients. Lin et al. used autologous dendritic cells incubated with LMP2 peptides to vaccinate patients with NPC; after four injections CD8 T-cell responses to LMP2 were induced by the peptides, which correlated with tumor regression in two of nine patients[14]. Chia et al. transduced autologous dendritic cells with an adenovirus vector expressing a truncated form of LMP1 and full length LMP2. Injection of 16 patients with metastatic NPC with the dendritic cells resulted in a partial response in one patient and stable disease in two patients, although no expansion of LMP1 and LMP2-specific T cells was detected in vivo[15].

Future directions in immunotherapy

Patients who have recurrent NPC were exposed to multiple lines of chemotherapy and repeated irradiation which will decrease their white cell counts and lead to lymphopenia. It is not always possible to expand enough CTL for treatment in these heavily pre-treated patients. As shown in our previous study, the success rate of T cell expansion was only 72.7%. One way to get around this problem will be by use of allogeneic immune transfer. Allogeneic HLA-matched EBV specific T cells have been used for the treatment of EBV-associated lymphomasiv. It will be important to explore the potential of allogeneic EBV-specific T cells for patients with NPC. HLA types among Chinese are limited and quite homogenous. The success of T cell expansion from healthy adults is likely to be higher and this will make the treatment more efficient and more readily available and potentially be an "off-the-shelf" treatment for recurrent NPC.

Active immunization with the AdE1-LMPpoly vector as a vaccine can be considered. Patients with raised EBV DNA after primary concurrent chemotherapy for NPC are at high risk of further relapse[16]. Additional treatment is warranted in this group of high risk patients. The NPC 05-02 study in Hong Kong and the ongoing RTOG HN001 study are evaluating the effectiveness of adjuvant chemotherapy in this group of patients. However, it is not always easy for patients to tolerate more intensive chemotherapy after concurrent chemoradiotherapy. Immunotherapy is worth exploration in this group of high risk patients. Both adoptive immune transfer in an adjuvant setting or by using the AdE1-LMPpoly vector as a prophylactic vaccine for prevent of recurrence would be worth further study.

Immune checkpoint blockade with antibodies that target cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and the programmed cell death protein 1 (PD-1/PD-L1) has proven effectiveness in treatment of melanoma and demonstrated promise in a variety of malignancies. Programmed death-1 (PD-1), belonging to the CD28 family of proteins, is a surface receptor of T-cell which regulates cellular proliferation and metabolism. The ligand called programmed death-ligand 1 (PD-L1) has been discovered to overexpress commonly in many solid tumours including non-small cell lung cancer, renal cell carcinoma, melanoma, colorectal cancer and prostate cancer etc. Binding of PD-L1 to PD1 leads to T-cell apoptosis and senescence and inhibition of this PD-L1 and PD1 interaction can potentiate the anti-tumour activity of T-cell. There was evidence that PD-L1 expression in NPC is induced and upregulated by LMP1[17]. We evaluated PD-L1 expression on both tumour cells and tumour-infiltrating lymphocytes in 126 NPC cases with immunohistochemical study (unpublished data). In our preliminary result, we noticed PD-L1 positivity in 20.0% of patients (21 out of 126 patients) with at least 25% tumour cells expressing PD-L1 positivity and 57.1% (72 patients) with at least 5% tumour cells expressing PD-L1 positivity. Interestingly about 5% tumour infiltrating lymphocytes also expressed PD-L1 positivity. Our study revealed that expression of PD-L1 is present in NPC. As previous studies on immunotherapy for NPC have shown response, it would be a good target to evaluate for the effectiveness of immune checkpoint inhibitors in NPC. Many pharmaceutical companies are developing different immune checkpoint inhibitors and clinical trials are performed in a wide range of solid tumors. It is important not to miss out NPC in these trials. Also, blocking both the LMP1 oncogenic pathway and PD-1/PD-L1 checkpoints may be a promising therapeutic approach for EBV positive NPC patients. It will be interesting to combine adoptive immune transfer with immune checkpoint inhibitors as the performance of immune checkpoint inhibitors may be enhanced in the presence of restored T cell population and efficacy.

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Comparison of the clinical characteristics of normal tension glaucoma patients with pre-treatment intraocular pressures in the high-teens and low-teens

Abstract

To study differences in risk factors for patients with Low-teens Normal Tension Glaucoma (LNTG), defined as those with a maximum untreated intraocular pressure (IOP) ≤15mmHg at all visits, and patients with High-teens Normal Tension Glaucoma (HNTG), designated as those with an untreated IOP >15mmHg at any visit. We also compared their natural rates of progression.

Methods

A prospective cohort study with serial applanation tonometry and automated visual field assessment. Presence of systemic diseases was captured from a computerised database in Hong Kong.

Results

Four-hundred and seventy eyes from 470 subjects (108 LNTG, 362 HNTG; with mean IOPs of 11.8mmHg and 15.3mmHg, respectively) with 3 years of follow-up were analysed. LNTG was significantly associated with increasing age (relative risk [RR]=1.02 per year, 95% confidence intervals [CI] =1.01-1.05, p=0.018) and thinner central corneal thickness (CCT) (RR=1.43 per 30μm thinning, 95%CI=1.13-1.76, p=0.003) in multivariate analysis; presence of systemic hypertension (54.6% and 43.4% for LNTG and HNTG, p=0.039), renal failure, and absence of family history of open-angle glaucoma in univariate analysis. There were no significant differences in visual field mean deviation progression rate.

Conclusion

LNTG as a continuum of open-angle glaucoma, constituted one-fourth of this cohort, and it progressed at a comparable rate to HNTG. LNTG patients have vascular risk factors and face the unique challenge of an uncertain treatment risk-benefit ratio. LNTG deserves more attention and research.

Introduction

Normal-tension glaucoma (NTG) and primary open-angle glaucoma (POAG) belong to the same disease spectrum. Intraocular pressure (IOP), across the entire range from high to low, is likely to affect disease progression[1]. Nevertheless, it is important to know, whether NTG patients with pre-treatment IOP in the high-teens, may clinically differ from those with IOP in the low-teens. There is evidence of different mechanisms of damage between these two groups: visual field damage may be more dependent on pressures in NTG patients with higher IOP (between 15-21mmHg) than in those with lower pressures[2]. There was a significantly greater peripapillary atrophy inferior to the disc in NTG patients whose IOP was ≤15mmHg[3], and localized retinal nerve fibre layer (RNFL) defect was closer to the centre of macula in this group[4]. The correlation between regional RNFL, as measured by scanning laser polarimetry, and functional changes in high-pass resolution perimetry, was not identical in NTG patients with IOPs in the high-teens versus low-teens, which was suggested as evidence for difference in mechanisms of damage[5]. Vascular factors were all along presumed to have a role in glaucoma progression in addition to IOP. There is a lack of literature on the role of vascular factors in NTG patients with high-teens versus low-teens IOP. Hence, while a 30% lowering of IOP is currently the most effective treatment for NTG in general[1], we lack data as to whether such reduction in patients with low-teens baseline IOP is as effective and rational or not.

What is more, such IOP reduction in NTG patients who already have IOP in low-teens may prove difficult. Treatment response to glaucoma medications is less with lower baseline IOP[6]-[9]. In one report, patients with a baseline IOP >15 mmHg achieved significantly greater pressure reductions with latanoprost (mean reduction 3.7 mmHg), than those with a baseline IOP <15 mmHg (mean reduction 1.1 mmHg) at all follow-ups[9]. The role of surgery such as trabeculectomy in this situation is yet undetermined with a necessarily narrower safety margin and a delicate point of balance between adequate IOP reduction and complications from hypotony.

This study aims to see, firstly, whether there are differences in ophthalmic and systemic vascular risk factors in NTG patients with high-teens and low-teens untreated IOP. Secondly, we compare their natural rates of progression.

Methods

This was a prospective cohort study conducted at Hong Kong Eye Hospital, a university-based tertiary eye centre. Patients were prospectively recruited between years 2003-2006. The study followed the principles in Declaration of Helsinki and International Conference on Harmonisation - Good Clinical Practice (ICH-GCP) guidelines, and informed consent was obtained from all patients. The respective ethics committees approved the

study protocol [ClinicalTrials.gov Identifier : NCT00321386].

Definitions

NTG was defined as follows:

1. Six median untreated intraocular pressure (IOP) consistently less than 21 mm Hg, with no mor e than one reading of 23 or 24 mm Hg, and no single measurement greater than 24 mm Hg, similar to the Collaborative NTG Study[10]. At least two readings were taken at a different time of the day from the rest;
2. Open drainage angles on dark room gonioscopy;
3. Glaucomatous optic disc cupping and loss of neuroretinal rim;
4. Visual field (VF) defect as per published criteria for glaucoma:[11]
   - Glaucoma hemifield test outside normal limits;
   - Pattern standard deviation (PSD) with P-value <5% ;
   - A cluster of ≥3 points in the pattern deviation plot in a single hemi field with P-value <5%, one of which must have a P-value <1%.

   Any one of these criteria, if repeatable, was considered as sufficient evidence of a glaucomatous VF defect.
5. Absence of secondary causes for the glaucomatous optic neuropathy (e.g. previous trauma, steroids use, uveitis etc.).

VF progression was defined using criteria modified from the Collaborative NTG Study[11][12]: a defect was considered to have deepened or enlarged if 2 or more points within or adjacent to an existing scotoma had worsened by at least 10 dB, these 2 progressing points had to be adjacent. A new defect was also part of progression and was defined as a cluster of at least 3 points meeting the criteria for a visual field defect occurring in a previously normal part of the field. In addition, progression was also deemed to have taken place if at least one of the innermost 4 points showed at least 10 dB deterioration. Any field progression had to be verified on at least one subsequent field with no other explanation on clinical examination for deterioration. On each confirming field, the points showing progression need not have been the very same points, but in the same region, at least 50% of the points had to be the same, and the adjacency requirement remained unchanged[11][12].

Low-teens Normal Tension Glaucoma (LNTG) was defined as the maximum untreated office IOP ≤15mmHg in all serial visits. We designated those with untreated office IOP measurement >15mmHg in any visit as High-teens Normal Tension Glaucoma (HNTG).

Inclusion and exclusion criteria

We included NTG patients ≥ 18 years of age. If a subject had bilateral disease, the worse eye was recruited. Exclusion criteria were history of glaucoma treatment (e.g. IOP-lowering medication, glaucoma laser procedures or glaucoma surgery), history of previous ocular surgery (except for cataract surgery) and patients with diseases precluding accurate measurement of IOP, visual acuity (VA), RNFL thickness and VF tests.

Assessment for systemic conditions

The presence of the following systemic conditions was recorded: systemic hypertension (HT), hypotension, ischaemic heart disease (IHD), arrhythmia, diabetes mellitus (DM), hypercholesterolaemia, cerebral vascular accidents (CVA), migraine, obstructive sleep apnoea (OSA), sensorineural hearing loss and Raynaud's phenomenon. All diagnoses were obtained from history taking and confirmed by the clinical management system of the Hong Kong Hospital Authority, which was a computerised database connecting all public hospitals and clinics in Hong Kong.

Ophthalmic assessment

Ophthalmic assessment included best-corrected visual acuity (BCVA), IOP, slit lamp examination, dark room gonioscopy, disc assessment and dilated fundal examination. These measurements were performed at baseline and then at 4-monthly intervals for 36 months. VF examinations were performed at baseline and at 6-monthly intervals. IOPs were measured with Goldmann applanation tonometry. The median of three readings was used. Dark room gonioscopy was performed using a Goldmann 2-mirror gonioscope followed by indentation gonioscopy using a Posner lens as per established methodology[13]. The vertical cup-disc-ratio (VCDR) was taken as the longest vertical cup diameter divided by the longest vertical disc diameter. Estimates were made to the nearest 0.05. The vertical disc diameter was measured on the slit lamp using the Digital 1.0 lens (Volk Optical Inc., Mentor, OH, USA).

VF examinations were performed using static automated white-on-white threshold perimetry (program 24-2, SITA-fast, model 750, Humphrey Instruments, Dublin, CA) and the first reliable field was used in the analysis. Pachymetry was determined at baseline using an A-scan ultrasonic pachymeter (Pachette 500, DGH Technology Inc, Frazer, PA, U.S.A.). The median of five readings was taken as the central corneal thickness (CCT) as per our previous published protocol[14].

Follow-up

Patients were followed-up for 36 months. All patients were untreated unless there was evidence of field progression during the course of follow-up. In such case, treatment was aimed at IOP reduction of at least 30% from baseline.

Statistical analysis

All analyses were performed using SPSS for windows version 13.0 (SPSS Inc, Chicago, Ill). Continuous variables were expressed as mean (± standard deviation [SD]), categorical variables as individual counts and proportions. Normal distribution assumption was tested with the Shapiro-Wilk statistic. Univariate analyses were performed using independent sample t-test, Mann-Whitney-U test and Chi-square test as appropriate. Binary logistic regression analysis was performed to determine the significance of various factors to the presence of LNTG. Independent variables were chosen based on both empirical and statistical (from univariate results) association with the outcome variable. Likelihood ratio was employed for both entry and removal tests. The probability at entry and removal in the stepwise option were set at 0.05 and 0.10, respectively. The critical value of significance was set at $P < 0.05$ for all other analyzes.

Results

Initially 483 eyes of 483 NTG subjects were recruited. Except 13 subjects (2.7%), all completed a 36-month follow-up. These 13 eyes were excluded, leaving 470 eyes for analysis. Among them, 120 eyes came from a cohort in our another study[15]. Ninety-three percent of follow-ups were made within 3 months of scheduled day. Of the 470 eyes, 108 (23.0%) were classified as LNTG and 362 (77.0%) as HNTG. Their demographics and clinical characteristics are listed in **Table 1**. LNTG subjects were significantly older (mean age for LNTG and HNTG was 69.2 ± 11.7 and 63.7 ± 13.6 years, respectively, p<0.001); having significantly thinner CCT (for LNTG and HNTG that was 525.0 ± 31.0 μm and 540.4 ± 33.5 μm, respectively, p<0.001) and were less likely to be associated with family history of open-angle glaucoma (for LNTG and HNTG that was 1.9% and 8.6%, respectively, p=0.017). The mean VCDR for LNTG appeared to be smaller (p=0.002) on the basis that their mean vertical disc diameter was comparable to that of HNTG. This was reflected in the slightly better baseline MD and PSD indices for LNTG, though these two variables were not significantly different between LNTG and HNTG **(Table 1)**.

There were no significant differences in the prevalence of disc haemorrhage (DH), as well as the number of subjects showing visual field progression, between LNTG and HNTG **(Table 1)**.

The univariate analysis of association between LNTG and systemic diseases is tabulated in **Table 2**. LNTG was significantly associated with the presence of systemic hypertension (54.6% and 43.4% for LNTG and HNTG, respectively, p=0.039); and renal failure (3.7% and 0.8% for LNTG and HNTG, respectively, p=0.030).

The results of multivariate analysis are shown in **Table 3**. On binary logistic regression, advancing age (relative risk [RR]=1.02 per every year of increase, 95% confidence intervals [CI]=1.01-1.05, p=0.018), and thinner

CCT (RR=1.43 per every 30 μm of thinning, 95% CI=1.13-1.76, p=0.003), were associated with LNTG.

There were no significant differences in the rate of field progression between LNTG and HNTG **(Table 1)**. The rate of MD progression was -0.37 ± 2.78dB/year and -0.24 ± 1.69dB/year for LNTG and HNTG, respectively (p=0.378). The rate of PSD progression was 0.09 ± 1.18dB/year and 0.07 ± 1.00dB/year, respectively (p=0.714).

Discussion

In this consecutively recruited cohort, almost one-fourth (23%) of our subjects had LNTG. This suggests that, while LNTG should not be viewed as a distinct disease from HNTG, it constitutes a significant portion of our NTG population and may merit further research. Our study showed that there were a number of differences in various factors associated with LNTG versus HNTG. Advancing age was significantly associated with LNTG in multivariate analysis. Age is a known risk factor for having NTG[16], and its prevalence increases with age[16][17]. The implication is two-fold: firstly, our data suggested that age was a risk factor for glaucoma damage at the low side of IOP-continuum. One may postulate that advancing age may indicate a longer duration of exposure of optic nerve to various factors causing damage to RNFL even at this low IOP. There may be other yet unidentified factor(s) related to glaucoma damage that occurred with increasing age. Secondly, it may imply that LNTG patients presented themselves to our ophthalmic service at an older age. Presumably their remaining life expectancy may be shorter, and hence the risk-benefit ratio of filtration surgery in LNTG should be carefully considered, and would be expected to be different from a usual POAG patient in their 40's. In our cohort the median untreated IOP in LNTG was only 11.8 mmHg. To achieve the recommended 30% reduction would mean lowering the pressure to below 8.3 mmHg—this is not easy with eyedrops and not without risk with filtration surgery. Studies have shown that a 30% pressure reduction could only be obtained and maintained in about half of NTG patients with topical drugs, laser trabeculoplasty, or both[18]. For the remaining patients, surgical means such as trabeculectomy is likely to be required for 30% reduction. The potentially narrow safety margin and complications associated with trabeculectomy

Table 1. Demographics and clinical characteristics of patients with Low-teens Normal Tension Glaucoma (LNTG) and High-teens Normal Tension Glaucoma (HNTG)

	LNTG (n=108) (23.0%)	HNTG (n=362) (77.0%)	P Value
Age at recruitment (years)*	69.2 ± 11.7	63.7 ± 13.6	<0.001++
Female gender	55 (50.9%)	182 (50.3%)	0.906#
Median untreated office IOP (mmHg)*	11.81 ± 1.73	15.34 ± 2.41	<0.001+
Maximum untreated office IOP (mmHg)*	13.89 ± 1.67	18.90 ± 2.70	<0.001+
Minimum untreated office IOP (mmHg)*	9.89 ± 1.98	12.86 ± 2.64	<0.001+
Mean baseline best-corrected visual acuity (Snellen/ [LogMAR])	20/34 / [-0.225]	20/31 / [-0.189]	0.059+
Central corneal thickness (μm)*	525.0 ± 31.0	540.4 ± 33.5	<0.001++
Baseline vertical cup-to-disc ratio*	0.70 ± 0.12	0.74 ± 0.11	0.002+
Vertical disc diameter (mm)*	1.68 ± 0.17	1.68 ± 0.19	0.936+
Baseline MD (dB)*	-7.16 ± 6.12	-7.75 ± 6.99	0.643+
Baseline PSD (dB)*	5.03 ± 3.36	5.45 ± 3.79	0.583+
No. of subjects with field progression	35 (32.4%)	129 (35.6%)	0.537#
Rate of MD progression per year (dB/ year; minus sign denotes worsening)*	-0.37 ± 2.78	-0.24 ± 1.69	0.378+
Rate of PSD progression per year (dB/ year; plus sign denotes worsening)*	0.09 ± 1.18	0.07 ± 1.00	0.714+
Family history of open angle glaucoma	2 (1.9%)	31 (8.6%)	0.017#
Disc haemorrhage	19 (17.6%)	72 (19.9%)	0.596#

* Mean ± SD + Mann-Whitney U test ++Independent t-tests were performed # Chi-square test

The P value for Shapiro-Wilk statistic for age was 0.186 and that for central corneal thickness was 0.528. Independent t-tests were employed. The statistics for median, maximum and minimum untreated office intraocular pressure were 0.002, <0.001, and 0.009, respectively. Mann-Whitney U tests were employed for these variables.

LNTG = Low-teens Normal Tension Glaucoma, HNTG = High-teens Normal Tension Glaucoma, IOP = intraocular pressure, MD = mean deviation, PSD = pattern standard deviation, dB = decibel, LogMAR= logarithm of minimum angle of resolution.

Table 2. Comparing the profile of systemic diseases between Low-teens Normal Tension Glaucoma (LNTG) and High-teens Normal Tension Glaucoma (HNTG)

Presence of:	LNTG (n=108)	HNTG (n=362)	P Value
Mean number of systemic diseases*	2.0 ± 1.5	1.7 ± 1.4	0.098+
Systemic Hypertension	59 (54.6%)	157 (43.4%)	0.039#
Systemic Hypotension	4 (3.7%)	11 (3.0%)	0.730#
Ischemic Heart Disease	17 (15.7%)	50 (13.8%)	0.615#
Arrhythmias	11 (10.2%)	27 (7.5%)	0.362#
Diabetes Mellitus	21 (19.4%)	73 (20.2%)	0.869#
Hypercholesterolaemia	16 (14.8%)	54 (14.9%)	0.979#
Cerebrovascular accidents	11 (10.2%)	26 (7.2%)	0.309#
Migraine	13 (12.0%)	39 (10.8%)	0.713#
Obstructive sleep apnoea	1 (0.9%)	12 (3.3%)	0.184#
Hearing Deficit	1 (0.9%)	12 (3.3%)	0.184#
Blood loss requiring transfusion	5 (4.6%)	12 (3.3%)	0.521#
Raynaud's phenomenon	0 (0.0%)	3 (0.8%)	0.343#
Renal Failure	4 (3.7%)	3 (0.8%)	0.030#

* Mean ± SD + Mann-Whitney U test #Chi-square test
LNTG=Low-teens Normal Tension Glaucoma, HNTG=High-teens Normal Tension Glaucoma
Note: All percentages indicate % of LNTG or HNTG having the listed systemic condition

Table 3. Binary Logistic Regression Analysis for factors contributing to Low-teens Normal Tension Glaucoma (LNTG)

Risk Factors	Relative Risk	95% CI	P value
Age at recruitment (per every year of advancing age)	1.02	1.01 – 1.05	0.018
Central corneal thickness (per 30 µm of thinning)	1.43	1.13 – 1.76	0.003

Other variables significant in the univariate analysis were not significant in the multivariate analysis.
Omnibus tests of model coefficients revealed a good performance of the model ($p < 0.001$ for inclusion of each of the above variables).
IOP=intraocular pressure; LNTG=Low-teens normal tension glaucoma, CI=confidence interval

in this situation requires further prospective randomized studies and cost-effectiveness analysis on quality-adjusted-life-years.

Thinner CCT was significantly associated with LNTG in multivariate analysis. Currently a thin CCT is regarded as a risk factor for progression from ocular hypertension to POAG[19]. Despite the fact that thin CCT results in underestimation of IOP from Goldmann applanation[20], disease progression is thought not primarily related to such underestimation[21]. In our cohort, the difference in mean CCT between LNTG and HNTG was 15.4µm. However, the difference in median untreated IOP was 3.53mmHg. As there are currently no universally accepted reliable IOP correction formulae for CCT differences, we decided not to proceed with such adjustment. Nevertheless, a 3.53 mmHg difference would not have been explained in full by a 15.4 µm CCT difference even if existing IOP-correction formulae were applied[22].

In the Los Angeles Latino Eye Study (LALES), persons with thinner CCT had a significantly higher prevalence of POAG than did those with normal or thick CCT[21]. Studies have also shown that, CCT was thinner in NTG compared to POAG subjects[23]. Our study further suggested that NTG subjects with thinner CCT had a significantly higher prevalence of LNTG. CCT may be a surrogate for yet undetermined vulnerability factor that predisposes one to suffer from glaucoma damage even at a lower IOP.

While there were greater number of systemic diseases in LNTG (**Table 2**, p=0.098), this did not reach statistical significance, suggesting that such presumed vascular factors, in addition to the effect of pressure, were likely to be present in both LNTG and HNTG. We conclude that LNTG and HNTG are in a same continuum with overlapping aetiologies. Nonetheless, LNTG deserves discussion because of its unique management challenges.

Of note, both systemic hypertension and renal failure were significant for LNTG in univariate analysis. The correlation between arterial hypertension, hypotension and glaucoma were known[24][25]. How hypertension may play a part in NTG is yet incompletely understood. Reports have suggested that nocturnal hypotension to optic nerve head results in ischaemic damage, which may be caused by systemic beta-blockers[25]. One may postulate that this mechanism may be encountered more often in LNTG, and we suggest that, NTG patients, particularly those with LNTG, should consider co-management with physician to rule out chronic high or low blood pressure or nocturnal blood pressure dips. More studies on the relationship between renal failure, optic nerve head perfusion, and NTG, are also warranted.

A positive family history of OAG was significantly associated with HNTG in univariate analysis. This may indirectly suggest that LNTG has

less genetic influence or is genetically more heterogeneous than HNTG, with implications for future genetics studies.

Despite a slow rate of progression for our NTG subjects, it is important to note that the natural rates of progression, and the number of subjects who progressed, were similar between LNTG and HNTG. This underscores LNTG as an important entity. We did not treat our cohort before documenting their rate of progression, as we concurred with the conclusion from the Collaborative NTG study, that as not all untreated patients progressed, the natural history of NTG must be considered before embarking on pressure reduction[1]. In order to safeguard patients from being untreated from significant field progression, we employed the Anderson-Hodapp progression criteria which was a relatively sensitive one to capture very mild progression, so as to start treatment as soon as progression was confirmed. In our cohort, the annual MD progression rates were only -0.37dB and -0.24dB for LNTG and HNTG, respectively.

It is also interesting to note that there were no significant differences in the rate of occurrence of DH in both groups. This may either support a hypothesis that IOP levels had no direct correlation with DH, or a hypothesis that DH was unlikely to be a predominant cause for occurrence of LNTG. More studies are needed.

Our study has a number of limitations. We have not corrected for the dose effect of severity of various systemic diseases. A well-controlled medical condition is less likely to be significant as a risk factor for glaucoma damage. Subjects were Hong Kong Chinese and hence, generalizability to other ethnic groups may be limited. The duration of study was relatively short at 3 years. On the other hand, we believe the strength of this study lies in the prospective design, having a relatively large cohort, with high percentage of completion of study follow-ups, and documented the effects of risk factors free from influence of treatment.

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Calorie restriction prevents metabolic ageing caused by abnormal SIRT1 function in adipose tissues

Abstract

Adipose tissue is a pivotal organ determining longevity, due largely to its role in maintaining whole body energy homeostasis and insulin sensitivity. SIRT1 is a NAD-dependent protein deacetylase possessing anti-ageing activities in a wide range of organisms. The present study demonstrates that mice with adipose tissue-selective overexpression of hSIRT1(H363Y), a dominant negative mutant that disrupts endogenous SIRT[1] activity, show accelerated development of metabolic ageing. These mice, referred to as Adipo-H363Y, exhibit hyperglycemia, dyslipidemia, ectopic lipid deposition, insulin resistance and glucose intolerance at a much younger age than their wild type littermates. The metabolic defects of Adipo-H363Y are associated with abnormal epigenetic modifications and chromatin remodeling in their adipose tissues, as a result of excess accumulation of biotin, which inhibits endogenous SIRT1 activity, leading to increased inflammation, cellularity and collagen deposition. The enzyme acetyl-CoA carboxylase 2 plays an important role in biotin accumulation within adipose tissues of Adipo-H363Y. Calorie restriction prevents biotin accumulation, abolishes abnormal histone biotinylation, and completely restores the metabolic and adipose functions of Adipo-H363Y. The effects are mimicked by short-term restriction of biotin intake, an approach potentially translatable to humans for maintaining the epigenetic and chromatin remodeling capacity of adipose tissues and preventing ageing-associated metabolic disorders.

Introduction

Advancing age is associated with loss of energy balance and deterioration of metabolic functions. In older people, postprandial hyperglycemia is common[1]. The prevalence of glucose intolerance and insulin resistance increases progressively and significantly with age[2]. The redistribution of fat from subcutaneous depots to visceral compartment and from adipose tissues to ectopic sites plays a critical role in the development of ageing-related metabolic abnormalities, in particular type 2 diabetes. Older people exhibit elevated central fat deposition but decreased skeletal muscle mass [referred to as sarcopenic obesity][3]. Preadipocytes isolated from aged animals and humans have a reduced potential to differentiate and replicate, whereas aged adipocytes show a decreased capacity for lipid storage[4]. The mechanisms underlying age-associated changes in adipose tissue function and fat distribution remain incompletely understood.

SIRT1 is a mammalian ortholog most closely related to SIR2 (Silent information regulator 2), a protein identified in *Saccharomyces cerevisiae* that extends replicative lifespan[5]. Both SIRT1 and SIR2 belong to a conserved family of ageing regulators and are NAD-dependent deacetylases that catalyze the removal of acetyl groups from protein substrates[6]. In yeast, SIR2 acts as a transcriptional silencer by modulating histone modifications, especially at the mating-type loci and telomeric DNA regions[7]. These chromosome domains are analogous to the heterochromatin of multicellular eukaryotes. When a conserved histidine within the catalytic domain of SIR2 was converted to a tyrosine (H364Y), its histone modification activity was lost and the mutant acted in a strong dominant-negative manner[8]. Compared to SIR2, SIRT1 elicits a wider range of biological functions by interacting with and regulating diversified protein substrates[5].

Animal studies reveal that SIRT1 is a key energy sensor controlling cellular responses to nutrient availability and in turn protecting against ageing-related metabolic diseases[9]. SIRT1 is critically involved in the metabolic adaptation to fasting, exercise and calorie restriction[10], which up-regulate the activity of this anti-ageing protein in adipocytes and adipose tissues[11]. Activation of SIRT1 in turn stimulates fat mobilization and adiponectin production in adipose tissues[12-14]. Over-expression of SIRT1 in adipose tissues of mice induces a similar phenotype as calorie restriction [leaner and metabolically more active, with improved insulin sensitivity and glucose tolerance[14-16]]. This evidence suggest that adipose SIRT1 is a candidate drug target for promoting systemic energy homeostasis and healthy ageing.

Transgenic mice that selectively overexpress wild type human SIRT1 (hSIRT1) or hSIRT1(H363Y) mutant in adipose tissues have been established[14]. In the present study, the metabolic functions of these two types of transgenic mice, referred as Adipo-SIRT1 and Adipo-H363Y, respectively, are compared and related to those of the wild type (WT) controls. While Adipo-SIRT1 are more insulin sensitive and metabolically healthier than their WT littermates[14], Adipo-H363Y exhibit a phenotype of accelerated metabolic ageing. Calorie restriction has been applied to test whether or not this non-genetic intervention is able to restore a normal metabolic function in Adipo-H363Y. The results demonstrate that a large number of ageing-induced changes of gene expression in adipose tissues are prevented by calorie restriction, which significantly delays the development of insulin resistance, glucose intolerance and dyslipidemia in both WT and Adipo-H363Y. Limited biotin intake contributes to the beneficial effects of calorie

restriction on enhancing SIRT1 activity in adipose tissues and improving systemic energy homeostasis. At the epigenetic level, by preventing excessive histone biotinylation, calorie restriction facilitates SIRT1-mediated histone deacetylation and transcriptional silencing.

Methods and Materials

Animal experiments

All procedures were approved by the Committee on the Use of Live Animals for Teaching and Research (CULATR) of the University of Hong Kong and carried out in accordance with the ARRIVE as well as institutional guidelines for the care and use of laboratory animals. The generation of transgenic mice with selective overexpression of human SIRT1 (Adipo-SIRT1) or hSIRT1(H363Y) (Adipo-H363Y) in adipose tissues has been described[14]. Mice were housed in a room under controlled temperature (23 ± 1°C) and 12-hour light-dark cycles, with free access to water and standard mouse chow [4.07 kcal/g composed of 20% protein, 52.9% carbohydrate, 5.6% fat, 4.7% fiber, 6.1% minerals and vitamin mixture containing 0.3 mg/kg biotin; LabDiet 5053; LabDiet, Purina Mills, Richmond, IN, USA]. All animals appeared normal and were fertile, giving rise to healthy litters. Male mice were used for the present study.

The food intake for *ad libitum* fed mice was ~0.14g/g body weight/day. For calorie restriction, mice received 60% of the normal food supply (~0.08 g/g body weight/day) from the age of 10 to 40-week. To study the effects of biotin intake in the diet, mice were given either biotin-sufficient (4.11 kcal/g composed of 18% casein, 60% sucrose, 10% vegetable oil, 2% corn oil, 4% salt and vitamin mixture containing 0.45 mg/kg biotin; 02960407, MP Biomedicals, Santa Ana, CA, USA) or biotin-deficient (4.11 kcal/g composed of 18% casein, 60% sucrose, 10% vegetable oil, 2% corn oil, 4% salt and vitamin mixture without biotin; 02901030, MP Biomedicals) diets. Mice were randomly assigned to each experimental group (n=8-10) for diet treatment starting at the age of 10-week. Metabolic parameters were monitored on a weekly basis.

For in *vivo* RNAi treatment, three sets of chemically modified siRNAs for ACC2 were synthesized by RiboBio® (Guangdong, China), including Sense 5'– CCUACGAGAUGUUCCGUAAdTdT–3'/Antisense 3'–dTdTGGAUGCUCUACAAGG CAUU–5', Sense 5'–GCAUCAAGUAUGCUCUCAAdTdT–3'/Antisense 3'–dTdTCGUAGUUCAUACGAGAGUU–5'and Sense 5'–CCACCUAUGUGUACGACUUdTdT–3'/Antisense 3'–dTdTGGUGGAUACACAUGCUGAA–5'. Mice were treated with the mixture of ACC2 siRNAs (5 nmol/mouse/day) or the same amount of scrambled RNAi by intraperitoneal injection at six different sites for three consecutive days [17]. The epididymal adipose tissues were harvested 12-hour after the last injection for subsequent analysis.

Metabolic evaluations

Body weight, blood glucose and fat mass composition were measured between 10:00am to 12:00noon for mice that were either starved overnight or fed *ad libitum*. Blood glucose was monitored by tail nicking using an Accu-Check Advantage II Glucometer (Roche Diagnostics, Mannheim, Germany). The body composition was assessed in conscious and unanesthetized mice using a BruckerMinispec body composition analyzer (Bruker Optics, Inc., Woodlands, TX, USA). The intra-peritoneal glucose tolerance test (ipGTT) and insulin tolerance test (ITT) were performed as described [14] [18]. Circulating and tissue contents of lipids, including triglycerides, total cholesterols and free fatty acids were analyzed using LiquiColor® Triglycerides and Stanbio Cholesterol (Stanbio Laboratory, Boerne, TX, USA), and the Half-micro test kit (Roche Diagnostics), respectively. The fasting serum insulin concentration was quantified using the commercial ELISA kits from Mercodia AB (Uppsala, Sweden). Serum adiponectin and lipocalin-2 concentrations were determined with the in-house ELISA kit as described[19] [20]. Metabolic rate [VO_2, VCO_2, Respiratory Exchange Ratio (RER)] was measured by indirect calorimetry using a six-chamber open-circuit Oxymax system component of the Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA) as described [14]. All mice were acclimatized to the cage for 48-hour before recording the parameters during a two-day feeding and a 24-hour fasting period.

Microarray analysis

Total RNA was extracted from epididymal adipose tissues of WT, Adipo-SIRT1 and Adipo-H363Y mice aged 8 and 40-week using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Samples were derived from at least three mice in each group. The quality of RNA was assessed by a spectrophotometer (ND-1000, Nanodrop Technologies, Wilmington, DE, USA) and the RNA 6000 Pico LabChip with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The A260/A280 ratios of all RNA samples were between 1.9 and 2.1. The RNA Integrity Numbers were above 9. After reverse transcription, the labeled cDNA samples were hybridized to the Mouse Genome 430 2.0 expression arrays using the GeneChip® Fluidics Station 450 (Affymetrix Inc., Santa Clara, CA, USA). The intensity of the probed signals were scanned and captured by GeneChip® Scanner 3000 (Affymetrix Inc.). Quality of the raw intensity data was assessed using diagnostic plots by GeneSpring GX 12.6 (Agilent Technologies) and Affymetrix Expression Console (Affymetrix Inc.). Raw intensity values of gene expression were normalized and background-corrected using Robust Multi-array Average (RMA) algorithm. Genes with expression values lower than the 20th percentile of all intensity values were excluded from further analyses. The expression profiles of samples derived from WT, Adipo-SIRT1 and Adipo-H363Y were clustered and validated by principal component analysis. Data comparisons were performed between 1) 8-week old WT, Adipo-SIRT1 and Adipo-H363Y, 2) 40-week old WT, Adipo-SIRT1 and Adipo-H363Y fed *ad libitum*, 3) 8 and 40-week old *ad libitum* fed mice of the same genotypes, and 4) 8 and 40-week old mice of the same genotypes under calorie restriction. Differentially expressed genes were defined as more than a two-fold change using a generalized linear model. In total, a list of 3,915 genes that were changed in at least one set of the above comparisons was included in Supplementary Table 1 for further analysis. Venn diagrams were used for presenting the numbers of differentially or commonly changed genes. The MIAME-compliant microarray data have been submitted to and are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress, accession number E-MTAB-2777).

Quantitative reverse-transcription polymerase chain reaction (QPCR)

Quantitation of target genes was performed using SYBR Green PCR Master Mix (Qiagen) and an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). The primer sequences are listed in Supplementary Table 2. Data were calculated and are presented as relative expression of transcripts normalized to β-actin.

Chromatin immunoprecipitation (ChIP)-PCR

The primers for ChIP-PCR are listed in Supplemental Table 2. The histone acetylation and/or biotinylation at the promoter regions of different genes were analyzed and compared by ChIP-PCR as described with modifications [21]. In brief, epididymal adipose tissue (100mg) was minced into small pieces in cold phosphate buffered saline (137mM NaCl. 4.3mM Na_2HPO_4, 2.7mM KCl, 1.4mM KH_2PO_4, pH 7.4) with protease inhibitor cocktails. Formaldehyde was added to a final concentration of 1% and the mixture was incubated at room temperature for 15 minutes with moderate shaking. The crosslinking was stopped by adding glycine to a final concentration of 0.125M. The nuclei were isolated from the tissue lysates and re-suspended in lysis buffer (1% SDS, 50mM Tris-HCl, pH8.0, 10mM EDTA containing protease inhibitors) for sonication to obtain chromatin fragment of 500-1000bp. After preclearing with protein G-Sepharose beads [(Pierce, Rockford, IL, USA), blocked with 0.2mg/ml salmon sperm DNA and 0.5mg/ml bovine serum albumin], 50μg chromatin were incubated with 2μg antibody overnight at 4°C. Immune-complexes were collected on protein G-Sepharose beads (blocked as above), washed and eluted into 100μl ChIP elution buffer (1% SDS and 0.1M $NaHCO_3$). Half of the elutes were reverse

cross-linked and the other half diluted 1:10 with lysis buffer to perform the second ChIP with streptavidin-conjugated beads (Pierce) as described[22]. After elution with ChIP elution buffer, the crosslink was reversed by adding 8µl of 5M NaCl and incubating for 4-hour at 65°C. The DNA was purified by phenol-chloroform extraction. PCR amplifications were performed using 10% of the DNA samples and the resulting PCR products separated by agarose gel for image quantification by densitometry (MultiAnalyst Software, Bio-Rad). Quantitation was also performed by QPCR analysis. The results were normalized against those from either the input DNA samples or the first ChIP fractions and presented as fold changes.

SIRT1 activity measurements

SIRT1 activity was monitored using a Fluorometric Drug Discovery kit from Enzo Life Sciences International, Inc.(Plymouth Meeting, PA, USA). Fluorescence was measured with a fluorometric reader (CytofluorII 400 series, Applied Biosystems), whereby excitation was set at 360nm and emission detection at 460nm. The *in vitro* protein deacetylation assay was analyzed by high-performance liquid chromatography (HPLC) as described[14]. The histone H3 peptides, including ARTKQTARKSTGGK^{14}APRK^{18}QLC with or without acetylation at K^{14} and/or biotinylation at K^{18}, were used as substrates. In brief, recombinant SIRT1 (3.6µM) was incubated with peptide substrate (500µM) and NAD^+ (5 00µM) in 50mM Tris buffer (pH 7.5) at 37°C for 10 minutes, in the presence and absence of 500µM biotin. Trifluoroacetic acid (TFA) was added to 0.1% before fractionation by a reverse-phased HPLC on a Symmetry® C18 column (5µm, 3.9×150mm, Waters, Milford, MA, USA), using a 40 minutes linear gradient (0–40% buffer B containing acetonitrile with 0.5% TFA) at a flow rate of 1 ml/minute. Chromatographic peaks were detected by a UV detector at 214nm. The sizes of the peaks obtained by calculating the areas under the peaks were used for comparison.

Quantification of tissue biotin contents

The amount of total biotin (bound and unbound) was determined as described[23] with modifications. In brief, 30mg of tissue or cell lysates were hydrolyzed in sulphuric acid at 100°C for one hour. After neutralization, 3.5mg of activated charcoal was added to bind the liberated biotin. The mixture was centrifuged at 12,000rpm for 3 minutes and the bound biotin was released by adding 1ml of 5% ammonia ethanol solution. The supernatant was filtered and evaporated to dryness under nitrogen. The extract was reconstituted in 100µl methanol and mixed with 80µl of 0.1% 9-anthryldiazomethane (ADAM, w/v in ethyl acetate) for one hour at room temperature. The fluorescent biotin-ADAM was detected at an excitation wavelength of 365nm and emission wavelength of 412nm using the fluorometric reader. Biotin solutions (1-50ng/ml) were used to generate a standard curve for calibration and quantification.

Histological analysis

Paraffin sections (5µm) were prepared for adipose tissues collected from different groups of mice. Hematoxylin and eosin (H&E) staining was performed to analyze adipocyte histology under a light microscope (Olympus, Tokyo, Japan). The sizes of adipocytes were measured as described[18]. Masson's trichrome staining was performed using Accustain® Trichrome kits (Sigma Aldrich, St. Louis, MO, USA) to evaluate the extracellular deposition of fibrillar collagens.

Western blotting

Antibodies against total ACC, ACC2, histone H3, histone H4, acetylated histone H3 (K9) and acetylated lysine were purchased from Cell Signaling (Beverly, MA, USA). Antibodies against ACC1, SIRT1 and acetylated histone H4 (K5, K8, K12 and K16) were from Millpore (Billerica, MA, USA). Monoclonal antibodies against beta-actin and streptavidin-horseradish peroxidase (HRP) conjugate were obtained from Sigma Aldrich. For Western Blotting, proteins derived from cell or tissue lysates were separated by SDS-PAGE and transferred to polyvinylidenedifluoride membranes. After overnight blocking, membranes were probed with various primary antibodies followed by secondary antibodies. Immunoreactive antibody-antigen complexes were visualized with the enhanced chemiluminescence reagents from GE Healthcare (Uppsala, Sweden). For histone modification analysis, histones were extracted from the nuclear fractions of epididymal fat pads following a previously reported procedure[21]. Briefly, the pellets of nuclei were re-suspended overnight in 0.4M H_2SO_4 at 4°C. The supernatant was collected after centrifugation (16,000g, 10 minutes at 4°C) and precipitated with trichloroacetic acid at 4°C for one hour. The acid soluble histone fraction was subjected to 18% SDS-PAGE and visualized by Coomassie Brilliant Blue (CBB) staining. The biotinylated protein was detected by Western blotting using streptavidin-HRP conjugate and the acetylated protein detected with anti-acetyl-lysine antibodies.

Data analysis

The density of protein bands in Western blots were analyzed quantitatively using Image J 1.45 (NIH, Bethesda, MD, USA) software for calculating the expression ratios against loading controls. Representative Western blotting images are shown. Statistical calculations were performed with the Statistical Package for the Social Sciences version 11.5 software package (SPSS, Inc., Chicago, IL, USA). For multiple comparisons, the differences were analyzed by one-way ANOVA, followed by Dunnett's test. Differences in other comparisons were determined by an unpaired two-tailed Student's *t-test*. In all cases, statistically significant differences were accepted when *P values* were less than 0.05. All results were derived from at least three sets of independent experiments unless otherwise specified. Data are presented as mean ± SEM.

Results

Overexpression of hSIRT1(H363Y) in adipose tissue accelerates the development of metabolic ageing

The metabolic parameters, including body weight, blood glucose, serum insulin, and circulating lipid levels were monitored on a weekly basis. Systemic insulin sensitivity was evaluated by insulin (ITT) and intraperitoneal glucose (ipGTT) tolerance tests. Compared to both WT and Adipo-SIRT1, Adipo-H363Y showed a phenotype of significantly accelerated metabolic ageing (Figure 1). At as early as the age of 20-week, abnormal responses to ITT and ipGTT were detected in Adipo-H363Y when compared to age-matched WT (Supplementary Figure 1)[52]. At the age of 30-week, the responses of Adipo-H363Y to either insulin (ITT) or intraperitoneal glucose (ipGTT) tolerance tests were comparable to those of 60-week old WT (Figure 1A). By contrast, these responses in 60-week old Adipo-SIRT1 were not significantly different from mice at the age of 30-week. Although the body weight was not significantly different between the three groups of mice at the age of 12-week, Adipo-H363Y showed consistently higher gain of body weights than the other two groups of mice at other age points (Figure 1B). Fasting glucose levels of Adipo-H363Y were not significantly different from WT (data not shown). However, from the age of 40-week onwards, the non-fasting glucose levels were significantly higher in Adipo-H363Y than in the other two groups of mice (Figure 1C). Circulating insulin levels were rapidly rising with age, being higher in Adipo-H363Y by ~51%,~79% and ~85% than those of WT at the age of 20-week, 40-week, and 60-week, respectively (Figure 1D). The insulin concentration in 30-week old Adipo-H363Y reached a level similar to that in WT at 60-week of age. Circulating triglyceride levels augmented progressively in both Adipo-H363Y and WT, but remained the lowest in Adipo-SIRT1 (Figure 1E). Compared to WT, the development of ageing-associated hypertriglyceridemia was enhanced significantly by ~19%, ~21% and ~31% in Adipo-H363Y at 40, 50 and 60-week of age, respectively.

In line with their higher body weight gain, Adipo-H363Y showed significantly increased (by more than 10%) whole body adiposity from the age of 20-week onwards when compared to WT, whereas changes in opposite directions were observed in Adipo-SIRT1 (decreased by ~20%, Figure 2A). However, the percentage compositions of lean and fluid mass were

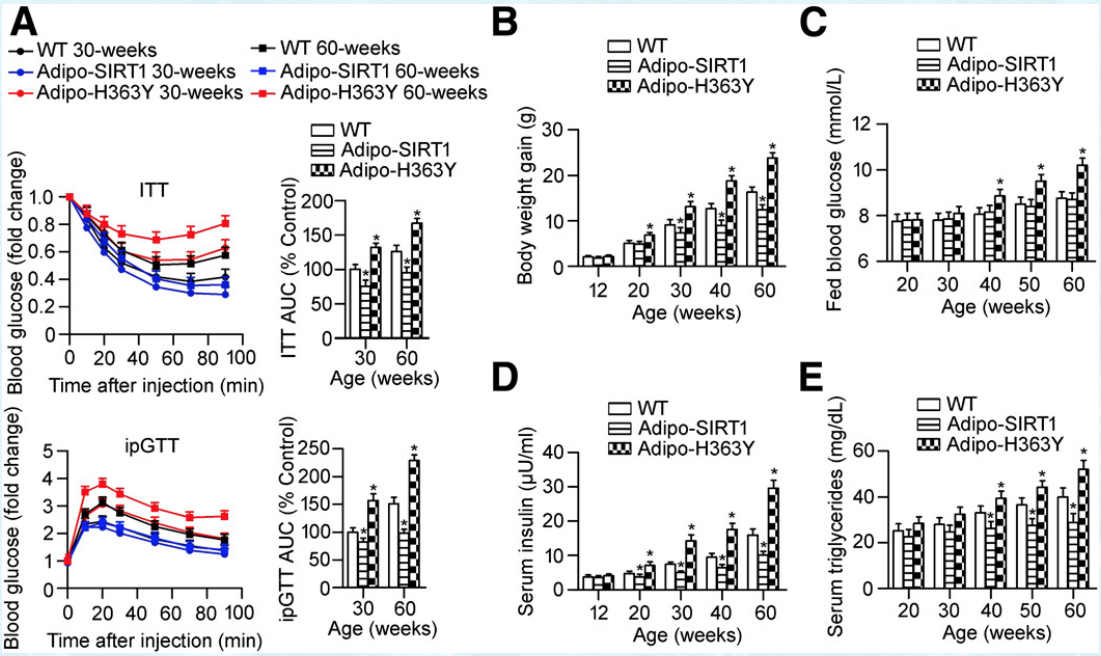

Figure 1—Overexpression of hSIRT1(H363Y) in adipose tissue accelerates the development of metabolic aging in mice. A: ITTs and ipGTTs were performed and compared between 30 and 60-week old WT, Adipo-SIRT1, and Adipo-H363Y. B: Body weight was recorded after overnight fasting and the body weight gain calculated by referring to those of WT, Adipo-SIRT1, and Adipo-H363Y at the age of 8 weeks. C: Blood glucose was recorded at 1000h in mice fed ad libitum. D: Circulating insulin levels were measured in sera collected from WT, Adipo- SIRT1, and Adipo-H363Y after overnight fasting. E: Mice were killed at different ages, and the fasting serum triglyceride concentration was measured. *P < 0.05, compared with the corresponding WT group (n=8).

not significantly different between WT and Adipo-H363Y (Supplementary Figure 2)[52]. On the other hand, the weights of visceral fat pads collected *post mortem* were not elevated in Adipo-H363Y when compared to those in WT (Figure 2B), indicating that the excess lipids were deposited in ectopic sites. Indeed, the triglyceride contents in liver and skeletal muscle of Adipo-H363Y were augmented significantly by ~35% and ~61%, respectively, when compared to WT (Figure 2C). In adipose tissues of Adipo-H363Y, the cholesterol and free fatty acid contents were significantly higher (by 1.5- and 1.9-fold, respectively) than in those of WT (Figure 2C). However, the size of adipocytes in epididymal adipose tissues of Adipo-H363Y was significantly smaller (Supplementary Figure 3)[52]. Histological assessment showed an increased cellularity and trichrome-positive streaks interspersed between adipocytes in adipose tissues of Adipo-H363Y (Supplementary Figure 4A)[52]. The expressions of genes involved in adipogenesis were significantly down-regulated, whereas those associated with inflammation up-regulated in adipose tissues of Adipo-H363Y (Supplementary Figure 4B)[52]. Indirect calorimetric analysis revealed that the respiratory exchange ratio (RER) was elevated significantly in Adipo-H363Y during both dark and light periods (Figure 2D). The increased RER in Adipo-H363Y was attributed mainly to the higher CO_2 production (VCO_2), but not to differences in O_2 consumption (VO_2).

Collectively, the results demonstrate that overexpression of hSIRT1(H363Y) selectively in adipose tissues of mice accelerates the development of insulin resistance, glucose intolerance, postprandial hyperglycemia and dyslipidemia, a cluster of metabolic defects that are closely associated with ageing.

Calorie restriction restores the metabolic functions of WT and Adipo-H363Y

Total SIRT1 protein expression was decreasing with age in adipose tissues of WT, but remained higher in those of Adipo-SIRT1 and Adipo-H363Y (Figure 3A). Compared to that of WT, SIRT1 activity was significantly augmented (by ~two-fold) in adipose tissues of Adipo-SIRT1but decreased (by ~60%) in those of Adipo-H363Y (Figure 3B). Compared to mice at the age of 12-week, SIRT1 activities were decreased by ~35%, ~10%, and ~55% in 40-week old WT, Adipo-SIRT1 and Adipo-H363Y, respectively. Calorie restriction [60% of *ad libitum* food intake] for 30-week not only prevented the down-regulation of SIRT1 activities, but also significantly enhanced the latter in adipose tissues of both WT and Adipo-H363Y (Figure 3C). At the age of 40-week, mice under calorie restriction showed similar levels of SIRT1 activity in their adipose tissues, which was elevated by ~2.1- and ~4.3-fold compared to the corresponding *ad libitum* fed WT and Adipo-H363Y, respectively. Calorie restriction significantly reduced the non-fasting glucose levels in all mice (Figure 3D), and improved the responses to ITT and ipGTT in both WT and Adipo-H363Y (Figure 3E). The area under the curve (AUC) values of ipGTT and ITT in calorie-restricted WT and Adipo-H363Y were not significantly different from those of age-matched Adipo-SIRT1 (Figure 3E, bottom panels). At the age of 40-week, the body weight gain and plasma insulin levels in the three groups of mice under calorie restriction were not significantly different (Figure 3F), and comparable to those in 20-week old WT (Figure 1, B and D).

Calorie-restricted WT, Adipo-SIRT1 and Adipo-H363Y exhibited similar diurnal rhythmic patterns of RER, ~0.82 in dark cycles and ~0.75 in light cycles (Figure 4A). The VCO_2 of Adipo-H363Y was significantly decreased by calorie restriction in both dark (~14%) and light (~23%) cycles, to comparable levels of those in WT and Adipo-SIRT1 (Figure 4A). The whole body adiposity was similar in the three types of mice across all age groups under calorie restriction (Figure 4B). The percentage weights of *post mortem* visceral fat pads in calorie-restricted mice at the age of 40-week were comparable to those in 20-week old mice fed *ad libitum* (Figure 4B and 2B). The ageing-induced augmentation of circulating triglyceride levels was prevented by calorie restriction in WT and Adipo-H363Y (Figure 4C). Calorie restriction significantly reduced the lipid contents in adipose tissues of all mice groups (Figure 4D), and attenuated the ectopic lipid deposition

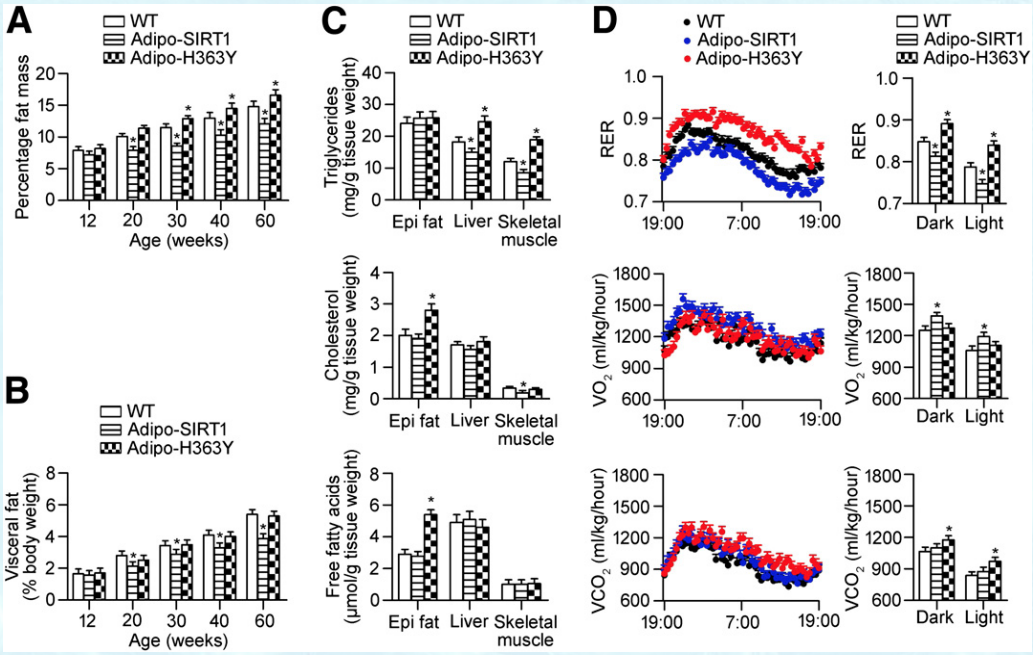

Figure 2—Selective overexpression of hSIRT1(H363Y) in adipose tissues leads to augmented body fat mass and ectopic lipid accumulation. A: Body fat composition (fat content/body weight %) was analyzed and compared in WT, Adipo-SIRT1, and Adipo-H363Y from different age-groups. The results are calculated as percentage values of the body weights. B: The wet weights of epididymal and perirenal fat pads were recorded and combined to calculate the percentage amounts of visceral fat compared with the total body weight. C: Triglycerides (upper), cholesterols (middle), and free fatty acids (bottom) were measured in epididymal (epi) fat, liver, and skeletal muscle collected from WT, Adipo-SIRT1, and Adipo-H363Y (40-week old, fasted for 16h). D: Indirect calorimetry analysis was performed to obtain the RER, VO2, and VCO2 values in WT, Adipo-SIRT1, and Adipo-H363Y (30-week old, fed ad libitum) (light period 0700–1900 h). *P < 0.05, compared with corresponding WT (n=10).

in Adipo-H363Y (Figure 4E).

Together, these findings suggest that despite the decreased SIRT1 activity in adipose tissues of Adipo-H363Y, calorie restriction can correct both local and systemic metabolic defects caused by adipose overexpression of the mutant hSIRT1(H363Y) and/or ageing.

Increased histone biotinylation causes abnormal chromatin remodeling in adipose tissues of Adipo-H363Y

Microarray analysis was performed using RNA samples extracted from adipose tissues of mice at the age of either 8-week (referred to as 'young'; when metabolic functions were comparable among the three groups) or 40-week (referred to as 'old'; when significant differences in metabolic functions were obvious). Principal component analysis of the adipose transcriptome revealed that samples derived from young WT, Adipo-SIRT1 and Adipo-H363Ycould be separated into three distinct clusters (Supplementary Figure 5A)[52]. The number of differentially expressed (by >two-fold) transcripts was obtained by pairwise comparison between mice of the same age group or between young and old mice of the same genotype (3915 genes in total; Supplementary Table 1). Compared to young WT, 1390 and 429 genes were differentially expressed in adipose tissues of age-matched Adipo-SIRT1 and Adipo-H363Y, respectively (Supplementary Figure 5B)[52]. Over 90% (1269) of the altered gene expressions in young Adipo-SIRT1 were due to up-regulation (Supplementary Figure 6A)[52]. In adipose tissues of Adipo-H363Y, genes related to cholesterol biosynthesis were significantly up-regulated and those of mitochondrial fatty acid oxidation significantly down-regulated (Supplementary Figure 6B)[52]. With the progression of ageing, different patterns of changes in adipose gene expression were observed in the three types of mice (Figure 5A). In general, the genome expression and chromatin state in adipose tissues of Adipo-H363Y was more rigid than the other two groups of mice. The average gene expression levels in adipose tissues of young WT, Adipo-SIRT1 and Adipo-H363Y were 0.210, 0.746 and 0.061, respectively. Compared to the corresponding young mice, a significant decrease was observed in the average gene expression level of old Adipo-SIRT1 (-0.081), whereas that of old Adipo-H363Y decreased slightly to 0.041 (Figure 5A). Compared to those of WT (1107) and Adipo-SIRT1 (2425), less (901) numbers of genes were changed by ageing (>two-fold) in adipose tissues of old Adipo-H363Y (Supplementary Figure 7, top panels)[52]. In addition to those involved in sterol lipid metabolism, genes encoding extracellular matrix and tight junction proteins were further up-regulated in old Adipo-H363Y (Supplementary Figure 7, bottom panel)[52]. A larger number (2425) of genes were changed in old compared to young Adipo-SIRT1, with ~70% down-regulated, suggesting a more flexible and dynamic chromatin structure in adipose tissues of these mice (Supplementary Figure 7 and 8)[52]. Calorie restriction prevented ~45% and ~36% of ageing-induced gene changes in adipose tissues of WT and Adipo-H363Y, respectively. Especially, out of the 103 genes up-regulated in old Adipo-H363Y adipose tissues, 93 (~90%) was prevented by calorie restriction (Supplementary Figure 8, top left panel)[52]. Among those that remained unchanged (≤two-fold) during ageing, 888 and 1029 were altered by calorie restriction (>two-fold) in WT and Adipo-H363Y, respectively (Supplementary Figure 7, top right panel)[52]. About 47% and 60% of genes changed by calorie restriction (>two-fold) in WT and Adipo-H363Y, respectively, showed similar up- or down-regulated patterns of change with those in old Adipo-SIRT1 when compared to young Adipo-SIRT1 (Supplementary Figure 8, bottom panels)[52].

Histone modifications control the stability and flexibility/rigidity of chromatin structure and its remodeling[24]. Thus, histones were purified from adipose tissues of WT, Adipo-SIRT1 and Adipo-H363Y to analyze the modifications at the epigenetic levels. Western blotting showed that even at the young age 8-week old), the three types of mice contained different amounts of acetylated and/or biotinylated histones in their adipose tissues.

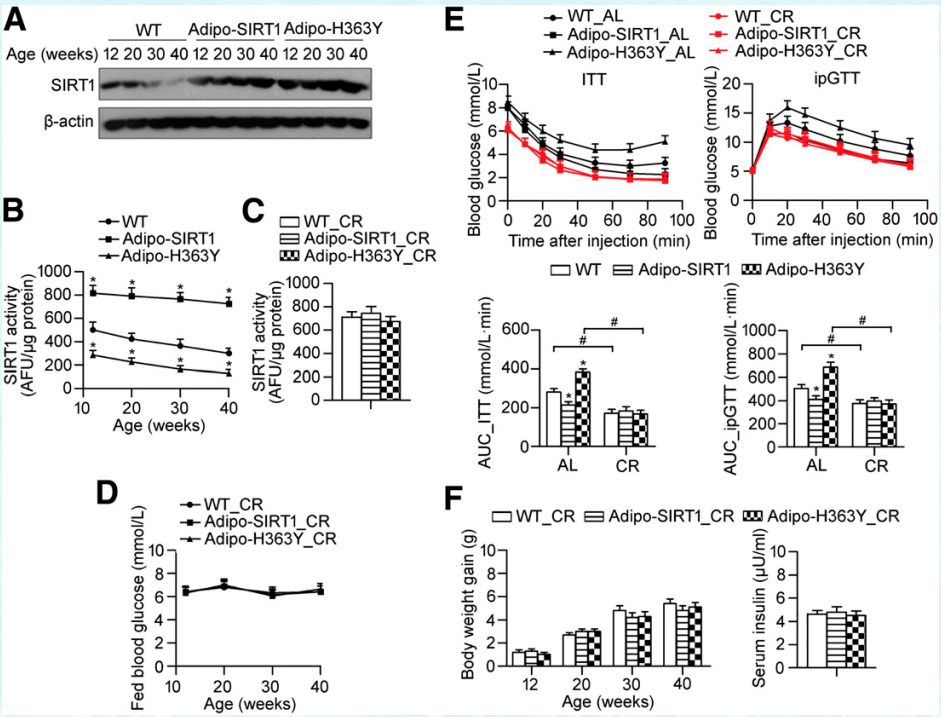

Figure 3—Caloric restriction prevents aging- and/or hSIRT1(H363Y) overexpression–induced metabolic defects in WT and Adipo-H363Y. A: Protein expression of SIRT1 was monitored in epididymal fat lysates of WT, Adipo-SIRT1, and Adipo-H363Y by Western blotting using an antibody recognizing both the murine and human species of this protein. B: A Fluorometric Drug Discovery Kit was used to measure the SIRT1 activity in epididymal fat pads collected from different age-groups of WT, Adipo-SIRT1, and Adipo-H363Y. For each sample, tissue lysates containing 20 mg of proteins were used. Fluorescent readings are presented as arbitrary fluorescence unit (AFU) in equal amount of proteins for comparison. C: SIRT1 activity was determined in epididymal fat pads collected from WT, Adipo-SIRT1, and Adipo-H363Y subjected to 30 weeks of calorie restriction (CR). D: Blood glucose was recorded at 1000h for mice under calorie restriction and compared across different groups. E: ITT (top left) and ipGTT (top right) were performed in ;30-week old WT, Adipo-SIRT1, and Adipo-H363Y either fed ad libitum (AL) or subjected to CR. The AUCs are compared (bottom left and right, respectively). F: Body weight gain was calculated as in Fig. 1B for WT, Adipo-SIRT1, and Adipo-H363Y subjected to calorie restriction from the age of 12 weeks onwards (left). Fasting (16h) insulin concentration (right) was measured using sera samples collected from 40-week old WT, Adipo-SIRT1, and Adipo-H363Y under calorie restriction. *P < 0.05, compared with the corresponding WT group; #P < 0.05, compared with the corresponding AL group (n=8).

Adipo-H363Y showed the highest level of histone biotinylation, whereas histone acetylation was significantly decreased in Adipo-SIRT1 (Figure 5B). Co-immunoprecipitation was performed and the results further confirmed that histones, including H3 and H4, were acetylated and biotinylated in adipose tissues of the three groups of mice, with acetylated histones containing significantly higher amounts of biotinylation (Supplementary Figure 9) [52]. Profile plots revealed that calorie restriction decreased the average gene expression levels in both old WT and old Adipo-H363Y (Figure 5C), which was in line with the reduced histone acetylation levels in both types of calorie restricted mice (Figure 5D). In addition, calorie restriction significantly decreased the biotinylation of histones in Adipo-H363Y preparations. By contrast, calorie restriction had no significant effects on histone acetylation and biotinylation in adipose tissues of Adipo-SIRT1, both of which were significantly lower than in those of Adipo-H363Y (Figure 5D).

To confirm the altered epigenetic modifications in Adipo-H363Y, a number of genes were selected for analysis of the associated histones using chromatin immunoprecipitation coupled with quantitative PCR (ChIP-PCR). The expression of these genes, including *CUGBP, GADD, TRDN and ADIPOQ*, was altered by ageing and/or overexpression of hSIRT1(H363Y) (E-MTAB-2777 in ArrayExpress). Histone biotinylation was detected at the promoter regions of all four genes and samples derived from Adipo-H363Y exhibited the highest levels of biotinylated histones (Supplementary Figure 10, left panels)[52]. Consistent with gene expression changes induced by calorie restriction, the amount of biotinylated histones was significantly reduced in samples from calorie-restricted WT, Adipo-SIRT1 and Adipo-H363Y (Supplementary Figure 10, right panels)[52]. The results further demonstrated that the amounts of biotinylated histones were significantly lower in fractions of non-acetylated than in those of acetylated histones (Supplementary Figure11 and 12)[52].

Taken together, these results suggest that augmented histone biotinylation contributes to the rigid chromatin structure in adipose tissues of Adipo-H363Y, which prevents the adaptive responses of genomic activation and silencing, thus leading to an accelerated ageing phenotype of these mice. The abnormal epigenetic modifications can be largely removed by calorie restriction.

Biotin deficiency prevents the development of metabolic dysfunctions in WT and Adipo-H363Y

During ageing, biotin is progressively accumulated in adipose tissues of mice and inhibits the deacetylase activity of SIRT1[14]. The biotin content in adipose tissues of Adipo-H363Y was augmented significantly (by ~two-fold) even at the age of 20-week when compared to those in WT (Figure 6A, left panel). Calorie restriction prevented the accumulation of biotin in adipose tissues of both WT and Adipo-H363Y (Figure 6A, right panel). To further validate the involvement of biotin accumulation in adipose tissues as a cause of systemic metabolic deregulation, WT and Adipo-H363Y were given either a biotin-sufficient or a biotin-deficient diet from10 to 20-week of age. This relatively short period of biotin deficiency did not induce obvious abnormalities, but significantly reduced the biotin content and ele-

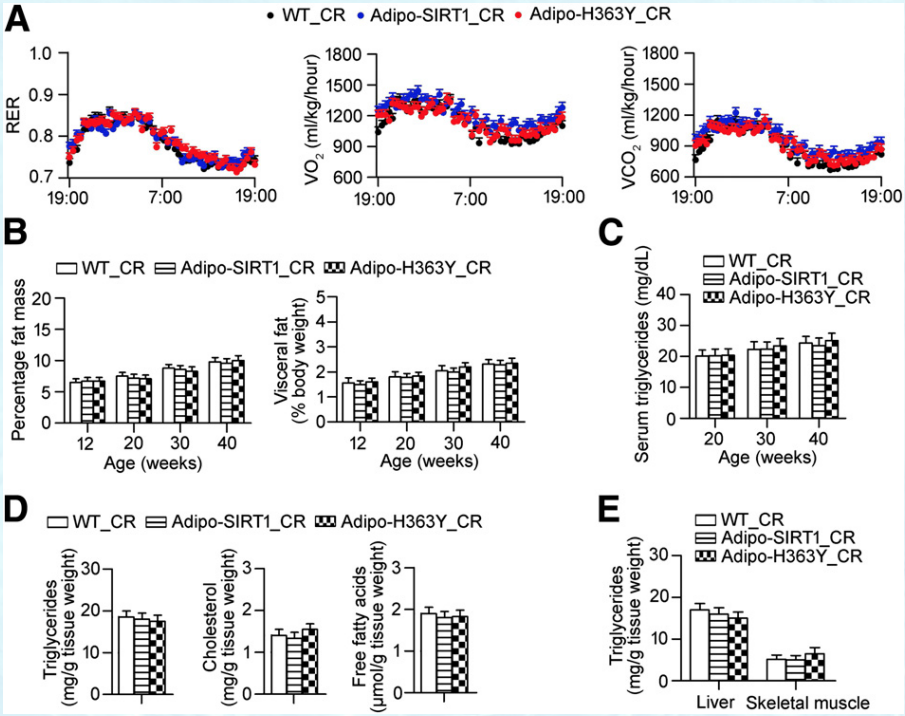

Figure 4—Caloric restriction restores the metabolic capacity and reduces ectopic lipid accumulation in Adipo-H363Y. A: Indirect calorimetric analysis was performed in 30-week old WT, Adipo-SIRT1, and Adipo-H363Y under calorie restriction (light period 0700–1900 h). B: Percentage body fat mass (total fat content/body weight %, left panel) and visceral fat (epididymal and per renal fat/body weight %, right panel) were measured and calculated for calorie-restricted WT, Adipo-SIRT1, and Adipo-H363Y as in Figure 2. C: Serum triglyceride concentration was monitored in calorie-restricted mice killed at the ages of 20, 30, or 40 weeks. D: Tissue contents of triglycerides (left), cholesterols (middle), and free fatty acids (right) were measured in epididymal fat pads collected from WT, Adipo-SIRT1, and Adipo-H363Y after 30 weeks of calorie restriction. E: Tissue triglyceride contents in livers and skeletal muscles collected from calorierestricted WT, Adipo-SIRT1, and Adipo-H363Ywere measured and compared (n=8).

vated SIRT1 activity in adipose tissues of WT and Adipo-H363Y (Figure 6B). Compared to those receiving the biotin-sufficient diet, the amount of acetylated and biotinylated histones was decreased significantly in adipose tissues of mice fed the biotin-deficient diet (Figure 6C). The latter diet also reduced the contents of the vitamin in livers [from 961 ± 113 to 703 ± 83 ng/g in WT and from 1042 ± 175 to 685 ± 92 ng/g in Adipo-H363Y]. By contrast, restriction of total calorie intake did not change the biotin contents in livers of either WT (710±132ng/g) or Adipo-H363Y (730±111 ng/g), which were comparable to those of mice fed *ad libitum* (651±98ng/g and 680±88ng/g for WT and Adipo-H363Y, respectively).

Biotin-deficient mice exhibited significantly improved ipGTT and ITT responses compared to those receiving a biotin-sufficient diet (Figure 6D). There was no significant difference between WT and Adipo-H363Y. The plasma insulin level was reduced in both WT and Adipo-H363Y fed with biotin-deficient diet (Figure 6E, left panel). The non-fasting blood glucose was not different between WT and Adipo-H363Y receiving the biotin-sufficient diet, which contained higher amounts of sucrose (60%) and fat (12%) than the standard mouse chow (3.18% and 5.6%, respectively). Nevertheless, after *ad libitum* feeding with biotin-deficient diet, the plasma glucose level was reduced in both WT and Adipo-H363Y (Figure 6E, middle panel). Circulating triglycerides were also decreased by the biotin-deficient diet to similar levels in WT and Adipo-H363Y, when compared with their respective biotin-sufficient groups (Figure 6E, right panel). Likewise, the triglyceride content in skeletal muscle and liver was significantly reduced to comparable levels in biotin-deficient WT and Adipo-H363Y (Figure 6F, left panel). Adipo-H363Y under biotin-deficient diet showed an enhanced capacity of triglyceride storage in their adipose tissues. In addition, the free fatty acid contents of adipose tissues were significantly reduced by the biotin-deficient diet in both WT and Adipo-H363Y (Figure 6F, right panel). Both calorie restriction and the biotin-deficient diet increased SIRT1 protein expression in adipose tissues of WT, but did not change that of Adipo-SIRT1 and Adipo-H363Y (Supplementary Figure13A)[52]. The circulating adiponectin levels were elevated by both calorie restriction and the biotin-deficient diet, whereas the circulating levels of the pro-inflammatory adipokine lipocalin-2 were reduced by these treatments (Supplementary Figure 13, B and C)[52].

Taken in conjunction, the present results support the concept that biotin accumulation in adipose tissue is causally involved in the development of ageing-associated metabolic abnormalities, and thus that limiting the intake of biotin represents an effective way to enhance adipose SIRT1 function and prevent metabolic ageing.

Up-regulated acetyl-CoA carboxylase 2 contributes to the dominant negative effects of hSIRT1(H363Y) on inhibiting endogenous SIRT1

SIRT1 regulates the protein stability and acetylation status of acetyl-CoA carboxylase (ACC), a biotin reservoir [14] [25]. The protein expression of ACC was up-regulated in adipose tissues of Adipo-H363Y (Figure 7A). Further analysis revealed that the total protein level of isoform ACC2, but not ACC1, and that of acetylated ACC2 were augmented significantly in adipose tissues of Adipo-H363Y (Supplementary Figure 14A)[52]. Both SIRT1 and ACC2 were present in mitochondria isolated from the three groups of mice (Supplementary Figure 14B)[52]. Both calorie restriction and biotin-deficient diet decreased the total amount of ACC2 in adipose tissues of Adipo-H363Y (Figure 7B). The amount of acetylated ACC2 was also reduced by the treatments (Figure 7C). To confirm that ACC2 is involved in regulating the total amount of biotin, specific chemically modified ACC2 siRNAs were used to treat WT and Adipo-H363Y in *vivo*. After treatment, epididymal adipose

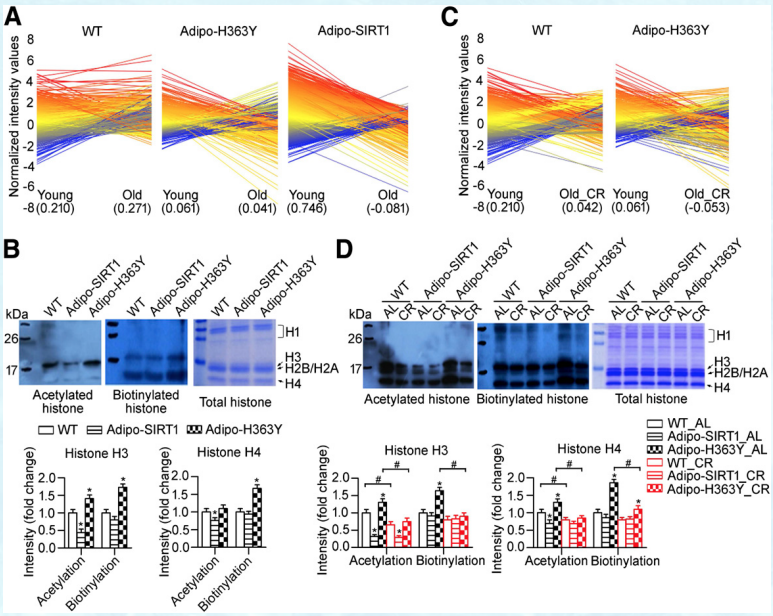

Figure 5—Calorie restriction prevents aging and hSIRT1(H363Y)-induced gene changes by inhibiting histone biotinylation and promoting histone acetylation. A: Profile plot was performed by GeneSpring GX 12.6 to display the normalized intensity changes between young (8-week) and old (40-week) WT, Adipo-SIRT1, or Adipo-H363Y. Each line represents a single gene of the 3,915 gene list summary (Supplementary Table 1)[52]. The average intensities of all genes were calculated for both "young" and "old" samples and are listed at the bottom of each graph. B: Histone enrichment was performed using epididymal adipose tissues collected from 8-week old WT, Adipo- SIRT1, and Adipo-H363Y. Western blotting was performed to analyze the global acetylated (top left, detected with antiacetylated lysine antibody) and biotinylated (top middle, probed with streptavidin-HRP) histone levels in isolated histone fractions (top right, stained with Coomassie Brilliant Blue). The band signals were quantified and presented as fold changes for H3 and H4, respectively (bottom left and right). C: Profile plot was performed as in Figure 5A to display the normalized intensity changes between young (8-week) and calorierestricted old (Old_CR, 40-week) WT or Adipo-H363Y. Each line represents a single gene of the 3,915 gene list (Supplementary Table 1)[52]. The average intensities of all genes were calculated and are listed at the bottom of each sample group. D: Epididymal fat pads were collected from mice (40-week old) that were either fed ad libitum (AL) or subjected to calorie restriction (CR). Global acetylated and biotinylated histone levels were determined as in panel B. *P < 0.05, compared with corresponding WT group; #P < 0.05, compared with corresponding AL group (n=3–6).

tissues were collected for analysis. Reducing total ACC2 protein levels significantly decreased the amount of biotin in adipose tissues of both types of mice, but increased their SIRT1 activities (Figure 7D). Moreover, ACC2 siRNA treatment significantly inhibited the biotinylation and acetylation of histones in adipose tissues of Adipo-H363Y (Figure 7E). In addition, recombinant adenovirus-mediated overexpression of hSIRT1(H363Y) in cultured 3T3-L1 adipocytes significantly increased the protein levels of ACC2, the intracellular accumulation of biotin, as well as the amount of biotinylated histones (Figure 7F).

Histones are the most conserved protein substrates of SIRT1[26]. To define the interrelationships between histone acetylation and biotinylation, a series of histone H3 peptides containing 21 amino acid residues with acetylated K14 and/or biotinylated K18 were synthesized (Supplementary Figure 15A)[52]. In *vitro* deacetylation assays were performed to compare SIRT1-mediated deacetylation of acetyl-H3 with that of biotinyl-acetyl-H3 (Supplementary Figure 15B)[52]. The ratios of nicotinamide (NAM)/NAD and deacetyl-H3/acetyl-H3, which were calculated based on the sizes of the peaks, were both significantly reduced (by ~80%) in reactions containing the peptide with biotinylated K18 (Supplementary Figure 15B)[52], indicating that biotinylated histones are less susceptible to SIRT1-mediated deacetylation. When biotin was present in the reaction buffer, SIRT1 did not cause deacetylation of acetyl-H3 peptide (Supplementary Figure 7C)[52]. In fact, the addition of biotin facilitated the formation of biotinyl-acetyl-H3 peptide clusters from acetyl-H3 peptide (Supplementary Figure 15C)[52].

These results demonstrate that up-regulated ACC2 in adipose tissues of Adipo-H363Y plays a major role in causing accumulation of biotin, which either directly inhibits the endogenous SIRT1 activity[14], or promotes histone biotinylation in turn preventing SIRT1-mediated deacetylation.

Discussion

Adipose tissue stores the surplus of energy as triglycerides and regulates whole body energy metabolism by secreting adipokines that elicit endocrine/paracrine functions. During ageing, the distribution and composition of body fat change significantly[27]. A decline of fat storage in adipose depots and an elevation of fat accumulation in ectopic organs (e.g. liver and skeletal muscle) contribute to the deterioration of systemic metabolic functions, including insulin resistance, hyperlipidemia and hyperglycemia[28]. Overflow of lipids into organs other than the fat depots is also a significant contributing factor to the accelerated ageing process in obese populations [29]. Calorie restriction is the most robust non-genetic approach to extend lifespan and improve health in mammals[30]. It counteracts obesity and related metabolic complications, by promoting weight loss and normalizing the function of adipose tissues[31]. In particular, calorie restriction increases the production of adiponectin, an insulin sensitizing adipokine[32], and represses inflammation in adipose tissues[33]. Yet, the molecular mechanisms underlying the beneficial effects of calorie restriction are still poorly understood. It has been questioned whether the actual decrease in calorie intake or the restriction of varying nutrients accounts for lifespan modulation by calorie restriction[34].

The present study demonstrates that restricting certain dietary components, in particular biotin, impacts positively on energy metabolism and prevents metabolic ageing, by enhancing SIRT1 activities in adipose tissues. Biotin and its metabolite, biotinyl-5'-AMP, are potent and competitive in-

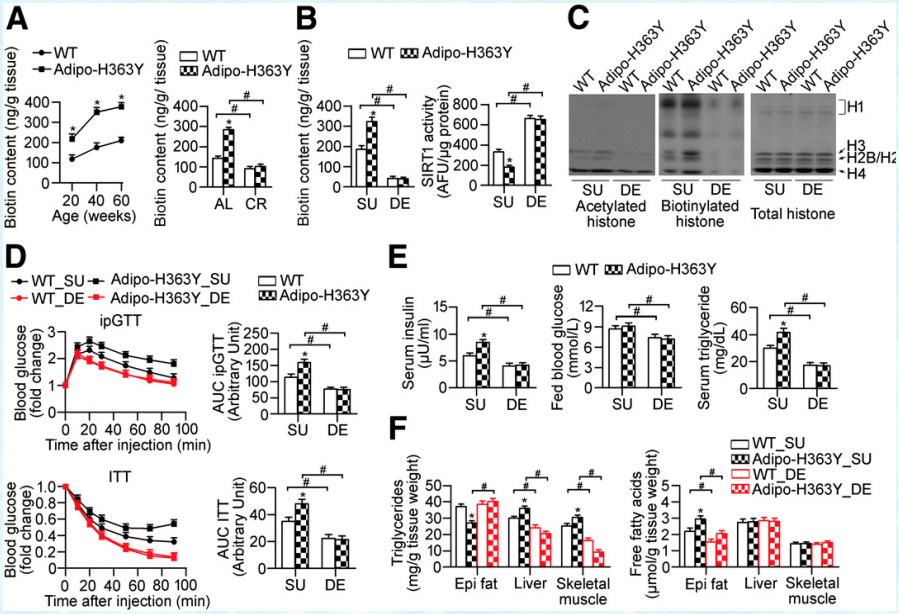

Figure 6—Biotin-deficient diet restores metabolic functions in WT and Adipo-H363Y. A: The tissue content of biotin was measured in epididymal fat pads collected from WT and Adipo-H363Y at different ages (left), and compared between mice (30-week old) that were fed ad libitum and under calorie restriction treatment (right). B: Biotin content (left) and SIRT1 activity (right) were measured in epididymal adipose tissues of WT and Adipo-H363Y subjected to treatments with biotin-sufficient (SU) or biotin-deficient (DE) diet. C: Acetylated or biotinylated histones were detected as in Figure 5 using adipose tissues collected from WT and Adipo-H363Y fed with either SU or DE diets. D: ipGTT (top) and ITT (bottom) were performed in WT and Adipo-H363Y subjected to 10 weeks of SU or DE diets. The AUCs were calculated and compared (right panels). E: Serum insulin, nonfasting glucose, and circulating triglyceride levels were measured and compared in WT and Adipo-H363Y fed with SU or DE diets. F: Tissue triglycerides (left) and free fatty acids (right) were measured in WT and Adipo-H363Y fed with SU or DE diets. *P < 0.05, compared with corresponding WT control group; #P < 0.05, compared with the corresponding SU group (n=8).

hibitors of SIRT1[14]. Biotinyl-5'-AMP occupies the NAD binding site and prevents the breakdown of NAD by SIRT1. Since NAD also acts as a co-factor permitting SIRT1 to interact with protein substrates, inhibition of NAD binding by biotinyl-5'-AMP prevents the interactions between SIRT1 and acetylated protein substrates[14]. Biotin occupies the binding pocket of nicotinamide, which may prevent the conformational change from non-productive to productive SIRT1[35] [36]. Calorie restriction reduces total biotin levels in adipose tissues; as a result, the direct inhibitory effects of this vitamin on SIRT1 activity in such tissues of calorie-restricted WT and Adipo-H363Y are alleviated.

The present results further suggest that biotinylation of protein substrates represents an indirect route by which biotin interferes with the enzymatic functions of SIRT1. Biotinylated histone peptides are resistant to SIRT1-mediated deacetylation. Although being relatively rare[37], biotinylated histones have been detected in human cells and play a functional role in gene silencing, responses to DNA damage, and cell proliferation[38]. Spontaneous histone biotinylation occurs in the presence of biotinyl-5'-AMP and in the absence of biotin protein ligases[39]. Thus, an increased intracellular biotin content and/or turnover can elevate the amount of biotinylated histones. So far, eleven biotinylation sites in histones have been identified, including K9, 13, 125, 127, 129 in histone H2A, K4, 9, 18, 23 in histone H3, and K8, 12 in histone H4 [40]. Biotinylated histones contribute to epigenetic modulations involved in the regulation of chromatin structure/stability and genome activation/inactivation. This conclusion is supported by the present observation that different levels of histone biotinylation determine the distinctive patterns of ageing-induced chromatin remodeling. Biotin-containing nucleosomes maintain a more condensed average structure[41]. In Adipo-H363Y, increased biotinylation prevents endogenous SIRT1-mediated deacetylation of histones and renders the genome more rigid even at a relatively young age. The resulting structural changes alter the chromatin activities of Adipo-H363Y, causing an imbalanced production of pro- and anti-inflammatory adipokines, abnormal expression of genes involved in lipid metabolism, adipogenesis and extracellular matrix deposition, and aberrant genetic adaptations to external conditions and ageing. However, calorie restriction can restore the nucleosomal remodeling process and reset the basal state of the genome structures in adipose tissues of Adipo-H363Y. The present study further demonstrates that the beneficial effects of calorie restriction can be mimicked by restriction intake of biotin, which directly [14] and indirectly (by modifying its protein substrates) enhances the endogenous activity of SIRT1.

The initial breakthrough of identification of SIR2 as an enzyme came along with the identification of the *Salmonella typhimurium* CobB protein, a SIR2 homolog[42]. CobB compensates for the lack of CobT mutants during vitamin B12 biosynthesis and possesses nicotinate mononucleotide (NaMN)-dependent phosphoribosy transferase activity. Thus, CobB catalyzes the release of nicotinic acid from NaMN, whereas SIRT1 removes nicotinamide from NAD. Biotin and nicotinamide were originally discovered as the same class of heat-stable vitamins[43]. Unlike nicotinamide, nutritional deficiencies of biotin are rare. Biotin functions in mammals as a CO_2 carrier for reactions in which a carboxyl group is transferred to one of four biotin-dependent carboxylases. Consequently, biotin participates as an important cofactor in gluconeogenesis, fatty acid synthesis, and branched-chain amino acid catabolism[44]. During ageing, the deacetylase activity of SIRT1 is decreasing progressively in adipose tissues, and this is accompanied by excessive biotin accumulation[14]. The evidence from the present study and a previous report suggest that biotin supply in diet dominantly inhibits SIRT1 activity and abolishes the beneficial functions of this anti-aging protein[14]. However, a self-defending mechanism for preventing excess accumulation of biotin is controlled by SIRT1 *via* ACC2, a major intracellular reservoir and a mitochondrial store of biotin[14] [45]. The present

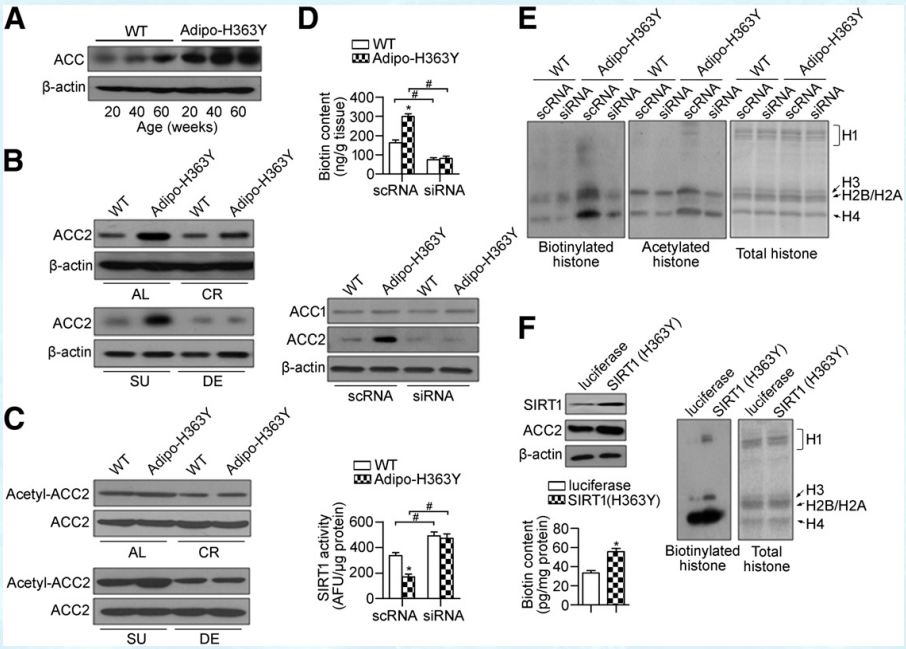

Figure 7—ACC2 regulates total biotin content in adipose tissues. A: Western blotting analysis of ACC protein expression in epididymal fat pads collected from WT and Adipo-H363Y at different ages. B: Expression of ACC2 was compared in epididymal fat pads of WT and Adipo-H363Y fed ad libitum (AL) or under calorie restriction (CR) (top), or subjected to biotin-sufficient (SU) or biotin-deficient (DE) diet treatments (bottom). C: Immunoprecipitation was performed using antibodies against ACC2 and the acetylated ACC2 was measured in the immunoprecipitates by Western blotting. D: Biotin content (top), ACC expressions (middle), and SIRT1 activity (bottom) were measured in epididymal fat lysates of WT and Adipo-H363Y treated in vivo with specific ACC2 siRNA or scrambled siRNA (scRNA). E: Biotinylated and acetylated level of histones were compared in epididymal fat pads of WT and Adipo-H363Y after RNAi treatment as in panel D. F: Murine 3T3-L1 preadipocytes induced to differentiate as previously described (50), followed by infection with recombinant adenoviruses encoding luciferase or the human SIRT1 mutant hSIRT1(H363Y) Q:4 at a multiplicity of infection of 100 (51). The cells were harvested 48h after infection. Protein expression of SIRT1 and ACC2 (top left), biotin content (bottom left), and biotinylated histones (right) were measured as above. *P < 0.05, compared with corresponding control groups; #P < 0.05, compared with the corresponding scRNA groups (n=3–4).

results suggest that by reducing the total protein amount of ACC2, SIRT1 can regulate the intracellular biotin contents in adipocytes. Under conditions of low biotin-intake, enhanced SIRT1 activity down-regulates ACC2, leading to a further decrease of intracellular biotin content and the elimination of biotinylated histones, in turn facilitating the dynamic chromatin remodeling and genome activation/inactivation.

The highly conserved H363 residue of SIRT1 forms the binding site for NAD. In yeast, the hSIRT1(H363Y) equivalent mutant SIR2(H364Y) possesses an impaired NAD-dependent histone deacetylase activity[7] [26]. The strong dominant-negative effects of SIR2(H364Y) has been suggested to result from the incorporation of an inactive SIR2 into silencing complexes. In mammalian cells, while the histone deacetylation activity of hSIRT1(H363Y) is impaired, the silencing effects of this mutant are not completely lost[46] [47]. The hSIRT1(H363Y) mutant exhibits defective substrate binding and catalytic activity that are not locus or species-specific[35]. The present study reveals that in adipose tissues of Adipo-H363Y, increased biotin contents and augmented biotinylation of histones play a dominant-negative role in preventing endogenous SIRT1-mediated deacetylation and chromatin remodeling. In adipose tissues of Adipo-SIRT1, the interactions between SIRT1 and ACC2 are significantly enhanced and more sensitive to nutritional changes. By contrast, mice with overexpression of hSIRT1(H363Y) in adipose tissues show significantly decreased interactions between ACC2 and SIRT1 (data not shown), leading to a persistent elevation of the protein levels of ACC2, both under basal conditions and during dietary interventions. The present results demonstrate that down-regulation of ACC2 [the mitochondrial form of ACC [48]] reduces the biotin content in adipose tissues and leads to a significantly decreased level of histone biotinylation. Previous radioactive tracer studies suggest that a significant portion of biotin is localized as bound forms in both nuclei and mitochondria[49]. While ACC2 and SIRT1 are both localized in mitochondria, the amount of SIRT1(H363Y) in this organelle is significantly reduced, which may be attributed to the augmented ACC2 protein levels in adipose tissues of these mice.

In summary, histone biotinylation was significantly enhanced in adipose tissues of Adipo-H363Y, causing deranged epigenetic modifications and chromatin remodeling, which contributed to the accelerated ageing phenotype in these mice. Calorie restriction reduces biotinylated histone levels in adipose tissues of Adipo-H363Y, allowing nucleosome deacetylation to occur in these mice at similar levels as in WT. However, unlike biotin-deficient diet, calorie restriction could not further improve most of the metabolic parameters beyond the levels of those in Adipo-SIRT1, which correlates with its milder effects on biotin reduction in mice adipose tissues. By contrast, the biotin-deficient diet more aggressively decreases biotin contents in adipose tissues of WT and Adipo-H363Y beyond those in Adipo-SIRT1, supporting the interpretation that short-term biotin restriction in the diet is more effective in improving metabolic function than a general restriction of total calorie intake. Thus, while biotinylation directly inhibits SIRT1-mediated deacetylation of histones, calorie restriction or a diet deficient in biotin prevents histone biotinylation independent of SIRT1, which subsequently facilitates gene silencing and prevent ageing (Figure 8). Based on this information, one could speculate that in mammals, caloric restriction may enhance SIRT1 activity by selective depletion of biotin storage

in adipose tissue, in turn preventing ageing-associated metabolic disorders and promoting lifespan extension.

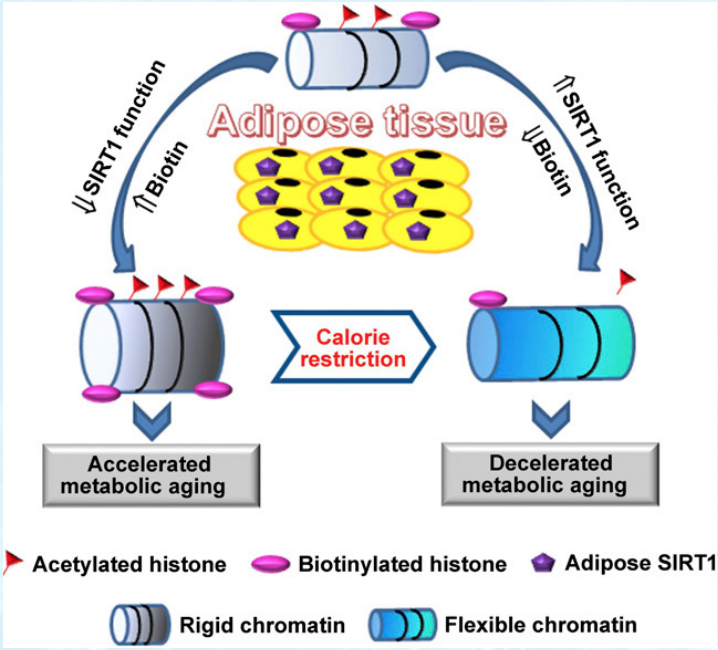

Figure 8—Schematic summary. Calorie restriction prevents SIRT1 dysfunction–induced abnormal chromosome remodeling and epigenetic modification in adipose tissues.

Acknowledgements

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All authors have no conflicts of interest to declare.

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Dr. Chun-fai Yu, Assistant Professor

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Efficacy of Waton sole patches on leg edema

Abstract

This controlled study aimed at assessing the efficacy of Waton sole patches applied on people with swollen legs or feet due to various diseases. 34 volunteer subjects were enrolled and they were randomized divided into treatment group (n=26) and control group (n=8). Every subject was required to apply the sole patch every night on their soles continuously for 8 nights. The sole patch efficacy was evaluated by three assessment methods: the percentage change in Numeric Pain Rating Scale scores (NPRS), thigh circumference measurement, and Subject Global Impression of Change (SGIC) from baseline to day 8. The results showed that the decrease in pain and swollen level from baseline on day 8 showed statistical significance ($P<0.05$). There was no application-site related burning or pain reported from all the subjects. Waton sole patches were safe to use and they reduced pain and swollen level in subjects with swollen legs or feet.

Introduction

The occurrence of swollen legs and feet are common amongst city dwellers. Some might be due to prolonged standing or sitting in the working environment, others may be due to leg injuries, obesity or certain diseases causing leg edema. Edema causes the swelling of tissues as a result of excess water accumulation whereas leg edema is a peripheral edema occurs in the feet and legs. There are some common diseases causing leg edema such as diabetes and high blood pressure[1][2].

In the marketplace, sole patches are also known as foot patches or detox foot pads, which are originally produced in Japan over 20 years ago. Most sole patches contain mainly plant materials (such as bamboo vinegar together with some other binding materials) enclosed in perforated non-woven polyester sachets. Each sole patch is applied onto the sole of each foot with a sticker while sleeping. After use, the sole patch material will become sticky as it extracts body fluid from the foot. Hence some sole patch manufacturers claim sole patches can promote natural detoxification of the body by extracting internal dampness and removing harmful wastes from the bodies. Moreover, a lot of benefits have been suggested after using soles patches, such as enhanced blood circulation, strengthened immune system and improved sleep quality.

In this study, Waton sole patch, one of the main sole patch brands available in local market, was selected to test its efficacy on pain relief and swollen level in people with swollen legs and feet was investigated. Voluntary subjects with swollen feet symptoms were invited to participate in this study by applying sole patches every night on their soles continuously for 8 days. The sole patch efficacy was evaluated by three assessment methods: the percentage change in Numeric Pain Rating Scale scores (NPRS), thigh circumference measurement, and Subject Global Impression of Change (SGIC) from baseline to day 8.

Our earlier open-label study showed that Waton sole patches could reduce pain and swollen level in both groups of subjects whose swollen legs and feet were caused by their various diseases and occupations. The sole patch efficacy was found to be slightly better in the latter group of subjects[3]. The current controlled study mainly focus on the efficacy of Waton sole patches on swollen legs and feet due to various underlying diseases of the subjects.

Materials and Methods

All the sole patches used in this study (both treatment and control groups) were Waton sole patches, provided by Waton International Limited, Hong Kong. The main ingredients in each treatment patch are bamboo vinegar powder which was made from natural Mousoudake bamboo grown in Japan with at least 15 ripening years and three filtration processes before packaging. A total of 34 voluntary human subjects (subjects thereafter) showing varying degree of swollen legs or feet symptoms (a minimum 3 years history of swollen leg symptoms) were invited to participate this controlled, double-blinded study with their written consent. The reasons of their swollen leg symptoms were diagnosed with different underlying diseases, such as diabetes, hypertension, renal diseases, rheumatoid or gouty arthritis. The subjects were divided randomly into treatment group (n=26) and control group (n=8). Both Waton patch and placebo patch (patch thereafter) had the same size and mass so that they were indistinguishable for both the investigators and the voluntary subjects. Wheat flour was used to replace the bamboo vinegar in the placebo patches for the control group of subjects.

Before the start of the study, the circumference (in mm) of the most swollen part of each subject's leg or foot was measured. The pain induced by the swollen legs or feet was assessed by a 0-10 Numeric Pain Rating Scale scores (NPRS), in which 0 = no pain, 1-3 = mild pain, 4-6 = moderate pain and 7-10 = severe pain[4]. All the subjects were required to apply a patch on each of their soles every night during sleep for eight hours continuously for 8 days. The position of the patch applied on the sole, which was divided into 3 zones (upper, middle and lower, Figure. 1), was determined according to the sole reflexology points related to their underlying reasons for swollen legs or feet[5][6]. For example, middle zone for a diabetic subject and lower zone for a subject with knee arthritis. After 8 hours of application, each patch was removed and the volunteers would return back to their normal daily activities until the next patch application at the following night. The swollen level measurement and pain assessment of each volunteer were recorded by two investigators employed in this study on Days 3, 6 and 8 until the completion of the whole study. Upon completion, each subject would complete a study questionnaire to reflect his or her SGIC (Subject Global Impression of Change), which reported how each of them felt before and

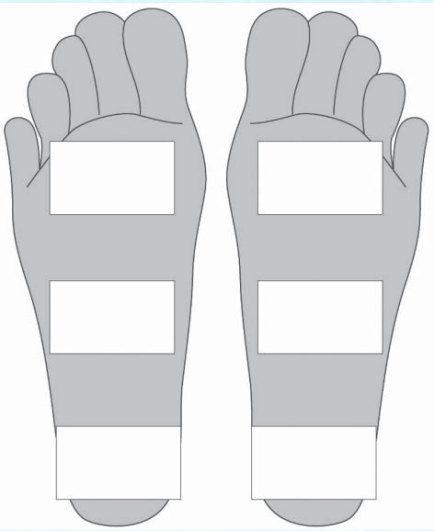

Figure 1. The position of the patch applied on the sole was divided into 3 zones (upper, middle and lower).

after the patch treatment on a scale between 3 to -3, in which -3 indicating "very much worse" to +3 indicating "very much improved", with 0 being "no change".

All subjects were required not to take any medication during the whole study. All measurements in this study were taken in triplicate and the results were expressed as mean ± standard deviation (SD) and the range value was taken between the minimum and maximum values. Statistical analysis of the assessment methods was determined by t-test and the statistical significance ($P<0.05$) was indicated.

Results

A total 34 subjects were enrolled and completed the whole study successfully. There were 26 and 8 subjects in the treatment and control groups respectively. The baseline demographic characteristics of all the subjects were shown in Table 1. All the subjects were adults and the average age was 48. 73% of the subjects were female. All the subjects were already diagnosed with different underlying diseases with a history of over 3 years before being enrolled in- this study. According to NPRS pain assessment, the subjects' pain ranged from mild to moderate (1– 6) with mean pain history of 8.2 years before the study. The thigh circumference measurement of the swollen legs ranged from 208–500mm.

The results (Table 2) showed that there was no significant statistical difference in the baseline data between the treatment and control groups. However, after 8 days, only the treatment group showed that the decrease in pain and swollen level from baseline to day 8 achieved statistically significant ($P<0.05$). In the assessment of NPRS and thigh circumference measurement, the relief in pain and circumference measurement achieved 64.5% and 2.8% from the baseline respectively. In the SGIC assessment, 84.6% of the subjects in the treatment group reported that their treatments were improved whilst the control group reported no improvement at all. At the end of the study, there was no application-site related burning or pain reported from all the subjects.

Discussion

Today, swollen legs and feet are very common city dwellers, properly due to their living environment or lack of physical exercise. Although some of the swollen symptoms are only temporary, many are caused by various types of diseases and there are always accompanied with pain in or near the swollen areas. This study demonstrated that Waton sole patches were safe to use and they reduced pain and swollen level in subjects with swollen legs or feet due to different diseases. The patches were usually used at night time during sleep. The patch would extract some body fluid from the swollen legs and became sticky itself. Most subjects reported that their sleeping quality was improved. Though Waton patch could not be considered as a replacement of medical treatment, it would be a complement to standard medical care in the amelioration of leg edema symptoms for health concerns.

Table 1. Baseline demographic characteristics of all volunteer subjects in this study

Volunteer subject	Treatment group (n = 26)	Control group (n = 8)	Total (n = 34)
Age (years), mean ± SD[a]	48.0 ± 19.56	58.6 ± 15.63	50.5 ± 19.08
Age range	21 - 76	39 - 80	21 – 80
Female, n (%)	19 (73.0)	7 (87.5)	26 (76.5)
Duration of pain (years), mean ± SD	6.6 ± 5.14	13.3 ± 6.36	8.2 ± 6.06
(range)	1 - 22	5 - 24	1 – 24
Baseline pain level, mean ± SD	4.3 ± 1.00	2.8 ± 1.49	3.9 ± 1.29
(range)	2 - 6	1 - 5	1 – 6
Baseline swollen level (mm), mean ±SD	328.0 ± 72.51	400.0 ± 99.68	344.9 ± 83.97
(range)	208 - 490	210 - 500	208 – 500

[a]SD – standard deviation

Table 2. Efficacy of Waton sole patch

Volunteer subject	Treatment group (n = 26)	Control group (n = 8)
NPRS[a] scores		
Baseline pain level, mean ± SD[b]	4.3 ± 1.00	2.8 ± 1.49
Day 8		
Actual, mean ± SD	1.5 ± 0.65*	2.5 ±1.31
Change from baseline (mean ± SD)	-2.8 ± 0.94	0.3 ± 0.89
Change from baseline (mean ± SD), %	-64.5 ± 17.96	6.25 ± 60.87
Subject with >60% decrease from baseline to Day 8, n (%)	21 (80.8)	0 (0)
Thigh circumference measurement		
Baseline swollen level (mm), mean ± SD	328.0 ± 72.51	400.0 ± 99.68
Day 8		
Actual, mean ± SD	318.8 ± 70.67*	401.3 ± 99.78
Change from baseline (mean ± SD)	-9.2 ± 5.62	2.5 ±5.35
Change from baseline (mean ± SD), %	-2.8 ± 1.81	0.3 ± 1.58
Subject with >1.5% decrease from baseline to Day 8, n (%)	21 (80.8)	1 (12.5)
SGIC[c]		
Day 8		
Improved, n (%)	22 (84.6)	0 (0)
Much improved, n (%)	18 (6 9.2)	0 (0)

[a]NPRS – Numeric Pain Rating Scale; [b]SD – standard deviation;

[c]SGIC – Subject Global Impression of Change (subjects reported how they felt before and after treatments on a scale -3 indicating "very much worse" to +3 indicating "very much improved", with 0 being "no change"); *Decrease in pain and swollen level from baseline on day 8 showed statistical significance (P<0.05).

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彭英昆

加拿大商頤安法恩傑國際有限公司（下稱「商頤」）（PharmEng Technology）亞洲區負責人。加拿大滑鐵盧大學（University of Waterloo）機械工程碩士、持加拿大專業工程師（Professional Engineer）執照、美國醫療法規學會（RAPS）醫藥法規專業認證、亞洲區醫療器械法規調和會（AHWP）技術委員會委員、國際製藥工程協會（ISPE）會員及技術審閱員、加拿大商會理事委員。彭負責的商頤公司於1997年在加拿大多倫多創立，並在美國、東南亞、大中華區等處設分公司，目前為加拿大最大之專業醫藥技術諮詢顧問公司，專注跨國專案計劃與執行、技術轉移等，在北美、南美、歐、非、東亞、南亞、中東等地區皆有實例經驗。

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國際市場准入：亞洲新興市場概述

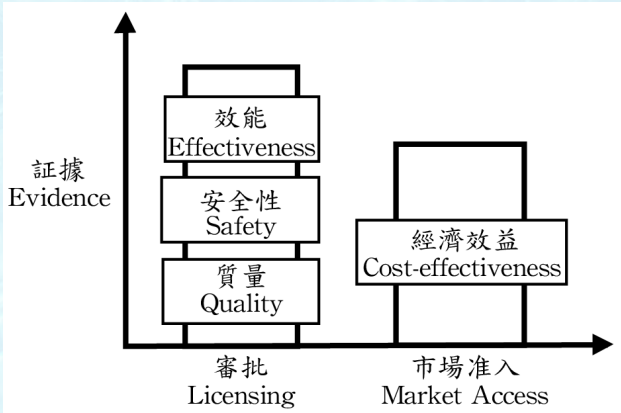
簡介

醫藥產業是最高度管制的產業之一。在亞洲，除了新興市場的醫療消費需求持續高度成長之外，法規及政策也在急速變化。

從技術面來講，醫療器械應秉持三大要素：安全性（Safety）、有效性（Effectiveness）、及品質（Quality）。從市場面來講，引用波士頓杜夫藥物發展研究中心（Tufts Center for the Study of Drug Development）科恩教授（J.P. Cohen）一說，「經濟效益（Cost-effectiveness）」已經儼然成為醫藥品上市的第四大要素（圖一）。

當前文獻不乏對於醫療器械技術面的詳細分析，但是多以歐美法規為基礎作討論，並且少有納入商業面的考量。如今，隨著各國積極在新興市場尋求成長，本文主旨在於結合技術面以及商業面的考量，對亞洲主要新興國家的市場准入相關法規以及其法規科學概念做一個導覽性的概述。

圖一、醫藥品上市的四大要素[1]



三大技術要素

從法規科學的觀點來看，沒有任何醫療器械能夠永遠的、絕對的安全。因此，醫療器械的管理必須是整個產品生命週期性的，並且在各生命週期階段對安全性、有效性及品質等三大技術要素作控管（表一）。

表一、三大技術要素在各產品生命週期階段的直接關係

	直接利害關係者	安全性	有效性	品質
研發	製造商	科學性的設計、臨床的安全性、相容性、安全與性能基本要求（Essential Principles）	科學性的設計、臨床的有效性、安定性、安全與性能基本要求（Essential Principles）	
生產	製造商			品質體系
包裝和標記	製造商	確保產品在運輸、存放、傳遞的過程安全；確保使用者有適當的使用資訊	確保使用者有適當的使用資訊	
廣告	經銷商	避免廣告資訊不實	避免廣告資訊不實	
販售	經銷商		確保經銷商有適當的專業資格	確保產品的存放合乎規格
使用	使用者	確保使用者有適當的使用資訊	確保使用者有適當的使用資訊	
棄貨	使用者	確保有危險性的產品不會對其他人、環境造成傷害		

具體的細節依當地市場法規而異。以國際性的產品為例，醫療器械法規全球協調組織（GHTF；已於2014年改組為國際醫療器械法規論壇，IMDRF，但現階段仍沿用GHTF技術檔）以及美國FDA為常見的國際參考規範。

原則上，歐盟、加拿大、美國、澳洲等西方前進國家之間也存在著法規的差異，但是在技術性的概念及架構差異已經不大。反之，亞洲新興國家之間仍然存在著相當大的分歧。稍後本文將進一步探討。

嶄新的醫療照護時代以及第四大要素

OECD國家的醫療支出從1980年代初期均GDP的7.3%，到2001年升到約8.4%[2]，可是接下來的10年速度快了一倍，成長到大約9.3%（2012）[3]。其中，美國的支出從GDP的8.7%（1980）成長到13.9%（2001）[2]，到了2012已達到17.0%[4]。然而，儘管支出持續上升，用於評估人口健康指數的預期壽命值並不見得有明顯提升（圖 二）[5]。

圖 二、健康調整預期壽命[4]

聚光燈回到東方，亞洲新興國家正在以驚人的速度追隨西方的醫藥品技術。醫藥品的法規迅速地在更新，試圖從體制上著手以促使產業升級、提升醫藥品的品質。中國的GMP從1988年到2011年進版了四次（在歐美，GMP的沿革大約花了40年的時間）。相較於先進國家推出新法時2到3年的公眾諮詢及緩衝期，亞洲新興國家往往縮短為1到2年。

除了產業之外，消費者以及政策單位也在追隨更高的醫療照護水準，但是卻帶來了步入先進國家後塵的隱憂。1995年至2012年間，新興市場的醫療支出平均以每年7.4%成長，遠高於GDP成長率的5.5%，到2022年前，成長率更預期會達到10.7%[5]。其中，中國的支出已從1995年時GDP的3.5%躍升到2012年的5.4%，馬來西亞2.8%升到4.0%，菲律賓3.4%升到4.4%，泰國3.5%升到4.5%，越南5.2%升到6.0%，印尼2.0%升到3.0%。儘管相較於OECD國家的9.3%仍有距離，但成長的速度卻是前所未聞。

第四大要素主要是針對有各種社會健康保險制度的市場，即儘管一個產品的技術規格合乎上市的三大要素，但是它是否符合個人或是社會的經濟效益？

亞洲區新興市場醫藥市場現況

表二顯示亞洲新興國家仍然充斥著不完全成熟的醫藥市場經濟環境。例如，儘管中國擁有最大的醫藥市場，它的規模並不與人口成為正比；世界第四大人口國的印尼，其醫療支出比例竟然低於人均GDP更少的越南及菲律賓。

表二、人口及醫藥市場資料[4][6]

市場	人口（百萬）（2013）	人口增長（2013）	GDP（PPP：美元）（2012）	GDP實際增長率（2013）	人均 GDP（PPP：美元）（2012）	預期壽命（年）（2013）	醫藥市場規模（美元）（2013）	醫療支出（%GDP）(2013)
新加坡	5	1.96%	$3230億	3.5%	$60,800	84	$8.4億	4.6%
馬來西亞	30	1.51%	$4950億	4.7%	$16,800	74	$31億	4.0%
泰國	67	0.52%	$6450億	3.1%	$9,500	74	$48億	4.6%
菲律賓	106	1.84%	$4200億	6.8%	$4,400	72	$34億	4.4%
越南	92	1.03%	$3360億	5.3%	$3,800	73	$32億	6.0%
中國	1351	0.46%	$12.2兆	7.7%	$9,100	75	$650億	5.6%
臺灣	23	0.27%	$8940億	2.2%	$38,400	80	$54億	6.6%[7]
香港	7	0.39%	$3660億	3%	$50,900	82	$70億	5.2%[8]
韓國	49	0.18%	$1.6兆	3%	$31,900	80	$150億	7.2%
印尼	251	0.99%	$1.2兆	5.3%	$4,900	72	$59億	3.1%

事實上，以中國為例，因為城鄉、體制等差異，從技術面來講，它屬於單一市場，但從商業面來講，它的各省市可能比較類似歐洲諸國的個別市場。

亞洲區新興市場對於醫療器械市場准入條件的現況

醫療器械法規架構總覽

韓國及臺灣一般被視為亞洲區法規最成熟、公信度最高的兩個市場，也是唯二有自己GMP體系的市場。但是各國之間差異仍然相當大。東協（ASEAN）會員國雖然簽署了以GHTF架構為基礎的東協醫療器械指引（ASEAN Medical Device Directive; AMDD），但是目前只有新加坡和馬來西亞已經開始執行。

因為分類體系的差異，介於分類灰色地帶的產品很有可能在不同市場變成不同分類。模糊界線產品（Borderline Product；在這裡指的是介於藥品於醫療器械界線的產品，它可能同時有藥品及醫療器械的特徵）的風險尤其大，以清單為基礎的分類體系多數無法對這些產品做定義。

表三、醫療器械法規架構總覽

市場	醫療器械法規	基礎架構	分類體系	品質體系（針對進口產品）
新加坡	有	GHTF、AMDD	GHTF（A、B、C、D）	ISO 13485、美國GMP
馬來西亞	有	GHTF、AMDD	GHTF（A、B、C、D）	ISO 13485、美國GMP
泰國	有	自有體系	醫療器械種類清單	ISO 13485、美國GMP
菲律賓	有	自有體系	醫療器械種類清單	ISO 13485、美國GMP
越南	有	自有體系	醫療器械種類清單	ISO 13485、美國GMP
中國	有	自有體系	風險分類（I、II、III）	ISO 13485、美國GMP
臺灣	有	美國式架構	風險分類（I、II、III）	臺灣GMP、ISO 13485、美國GMP
香港	無、自願性	GHTF	GHTF（A、B、C、D）	ISO 13485、美國GMP
韓國	有	歐盟式分類架構、美國式GMP管理架構	風險分類（I、II、III、IV）	韓國GMP、ISO 13485、美國GMP
印尼	有	自有體系	醫療器械種類清單	ISO 13485

上下游產業、上市前後管理

除了缺乏醫療器械法規的香港之外，所有市場都有不同程度的上市前註冊控管。但是，菲律賓、越南、和印尼缺乏完整的上市後監測體系。

新加坡的醫療器械良好分銷規範（Good Distribution Practice for Medical Devices；GDPMDS）是一個基於當地國情而提出的特別架構。因為多數醫療器械是進口的，許多傳統的經銷商實際上缺乏品質管制的專業，因此GDPMDS旨在提升當地經銷商的品質管制。學習新加坡經驗，馬來西亞也建立了類似的規範（GDPMD）。

表四、上下游產業、上市前後管理

市場	醫療器械註冊	製造商註冊	當地經銷商註冊	不良事件監測體系
新加坡	要	要	要、含GDPMDS	有
馬來西亞	要	要	要、含GDPMD	有
泰國	要	要	要	有
菲律賓	要	要	要	無
越南	要	要	要	無
中國	要	要	要	有
臺灣	要	要	要	有
香港	自願性	自願性	自願性	自願性
韓國	要	要	要	有
印尼	要	要	要	自願性

技術性內容

表五列舉一些關鍵的技術性要求。由於醫療器械的種類繁多，本文僅列舉關鍵的項目作為比較。例如，東南亞的泰國、菲律賓、越南、印尼等地對技術性的要求基本上缺乏明確規範，或是尚處於是否要遵循AMDD的灰色地帶，對於欲前往當地市場的外商都是一個不明確的風險。中國是唯一明言規範臨床必需在國內進行的國家。因為臨床需要的時間很長，如果在產品研發階段沒有規劃在中國進行臨床試驗，外加註冊的時間，造成「原產國已下市、中國還不能上市」的故事時有所聞。

表五、技術性內容

市場	安全與性能基本要求（EssentialPrinciples）	風險分析	生物相容性	臨床證據
新加坡	有具體規範	有具體規範	有具體規範	有具體規範
馬來西亞	有具體規範	有具體規範	有具體規範	有具體規範
泰國	無明確規範（視同AMDD）	無明確規範	無明確規範	無明確規範
菲律賓	無明確規範（視同AMDD）	無明確規範	無明確規範	無明確規範
越南	無明確規範	無明確規範	無明確規範	無明確規範
中國	有具體規範	有具體規範	有具體規範	有具體規範；臨床證據必須在中國執行
臺灣	有具體規範	有具體規範	有具體規範	有具體規範
香港	自願性、GHTF	自願性、GHTF	自願性、GHTF	自願性、GHTF
韓國	有具體規範	有具體規範	有具體規範	有具體規範
印尼	無明確規範	無明確規範	無明確規範	無明確規範

市場准入架構

表六顯示亞洲各國的醫療採購和定價體系，表七為報銷機制總覽。目前除了臺灣及印尼從稅收編列給單一給付者之外，其他國家有採用個人儲蓄、社區保險、強制保險、稅收補貼等機制。實務上，亞洲國家普遍存在著嚴重的城鄉距離，使得醫療基礎建設難以推廣到鄉間地區。全球第四大人口國的印尼為例，2010年的數據[9]顯示有高達63.5%的鄉間人口沒有受到保障（相較於中國的5.1%、日本0.0%、韓國0.0%、越南56.0%、馬來西亞0.0%、泰國3.0%等）。儘管印尼在2013年開始了全民健康保險，經濟學人在2014年的調查[10]發現，鄉間的空床率及給付率不然就是沒有資料、不然就是遠低於城市。再以中國為例，中國的職工醫療保險、城鎮居民基本醫療保險、和新農村合作醫療保險系統基本上是由中央定義架構，由地方省市自定採購、定價、報銷等額度、比例、上限等。因此，誠如稍早提到，但從商業面來講，中國的各省市實際上比較類似歐洲諸國的個別市場。

表六、醫療採購、定價體系

市場	公開招標	限制招標	競爭性談判	直接採購
新加坡	是			
馬來西亞	是		是	是
泰國		是		是
菲律賓		是	是	
越南	是			
中國	是	是		是
臺灣		是		
香港	是			是
韓國	是		是	
印尼	是			

表七、報銷機制

市場	全民健康保險	雙層共同負擔	強制保險	單一給付者	稅收為基礎之財務模式	社會健康保險	私人保險	社區健康保險
新加坡	是	是	是				是	
馬來西亞	是	是			是		是	
泰國	是				是		是	
菲律賓	是	是			是	是	是	
越南	是		是			是	是	
中國			是			是	是	
臺灣	是	是	是	是	是	是	是	
香港	是	是			是		是	
韓國	是		是				是	是
印尼	是	是		是	是	是	是	

案例研究：模糊界線產品

以模糊界線產品這個近年來備受矚目的產品類別為例，表八總結了亞洲各國法規框架的調查結果。可以說，以區域性的角度來看，法規的分歧可能是目前市場准入最大的挑戰。在缺乏專有法規的市場，模糊界線產品可能定義為藥品、可能定義為醫療器械、抑或無法可循。

表八、特殊產品案例

市場	模糊界線產品*	本地及進口產品差別法規	進口產品條件
新加坡	有專有法規框架可循	無	先進國家進口產品可依循簡化程式
馬來西亞	有專有法規框架可循	無	先進國家進口產品可依循簡化程式
泰國	無專有法規框架可循	無	先進國家進口產品可依循簡化程式
菲律賓	無專有法規框架可循	無	先進國家進口產品可依循簡化程式
越南	無專有法規框架可循	是	先進國家進口產品可依循簡化程式
中國	無專有法規框架可循	是	無
臺灣	有專有法規框架可循	是	先進國家進口產品可依循簡化程式
香港	無專有法規框架可循	無	只接受先進國家產品
韓國	有專有法規框架可循	是	先進國家進口產品可依循簡化程式
印尼	無專有法規框架可循	是	無

討論

如同普華永道的問卷調查結果，亞洲新興國家的最主要挑戰包含缺乏成熟的給付體系、不完整的醫療基礎體系、價格壓力（以及隱含的價值觀念問題）、法規等。又以法規及醫療體系來說，儘管許多國家架構上已近乎與國際同步，但實務上仍有極大的城鄉差距、對非先進國家的醫藥品有成文或不成文的限制等。這些挑戰不論對於產品所有權者、供應鏈關係者、以及政策者都造成區域性的整合困難。

不過，如同90年代以前的歐洲，在歐洲共同體設置中央程式（Centralised Prodecure）及相互采認程式（Mutual Recognition Procedure）之前，歐洲也有類似的法規分歧的問題。挑戰帶來契機，現在是否正是亞洲新興國家市場准入的契機點？

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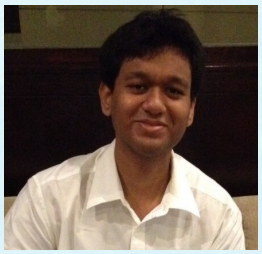

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Why waste time doing internships?

Make no mistake from the title, internships are important. We can't emphasize it any clearer. Internship experiences almost certainly gives anyone an advantage when searching for their desired job, even over your nerdy classmate, who wears a shiny golden medal engraved with the words, 'FIRST CLASS HONORS' on graduation day. Fortunately, we had the privilege of enjoying a meaningful and career-oriented internship and wish to share our insights and tips on how you, as a normal university student can take simple yet effective steps towards pursuing your dream internship.

Potential Nightmares:

Incoming internship opportunities are not favoured by everyone. The good oldays of late nights and endless slacking are over, the summertime madness has descended into sadness. Early morning alarms which you thought you left behind in college are back to haunt you. Welcome to the first day of your internship; where you find yourself tied up in formal clothes trying to sediment a good first impression. But amidst all this doom and gloom, you still remain optimistic; you're a working adult and will now be embarking on the first step towards fulfilling your dreams and ambitions. But then again, there is a flipside, why would you sacrifice your precious vacation time to work? After all, you're an intern, the company can operate effectively without you and must have shortlisted possibly the most mundane and menial jobs to delegate to you. At this moment, you'd take back your vacation time in a heartbeat but it's too late, you've already signed a commitment and find yourself horribly stuck. This dilemma is usual amongst university students, some pursue as they 'think' it might be a stepping stone to full-time employment whereas others are genuinely interested. All of this does beg the question, why waste time doing internships?

Problems encountered:

In search of the answer to the question, we conducted a small survey amongst 15 university students our age. According to our survey, some of the interns concluded that their internships failed to satisfy their expectations. Generally speaking, by taking part in the internship programme, interns are expected to exercise and display their talents in their specialities. However, due to their lack of working experience, they aspire to acquire more practical techniques to cope with real life working scenarios during the internship. Networking skills is a concern as well, they seek opportunities to build up their interpersonal network which they believe will be useful in their prospective career path.

Although interns might be willing to learn and work diligently, usually they do not have a clear sense about what they should do prior to the internship. Interns apply for internships normally with the purpose of gaining hands-on working experience, so as to enrich the contents in their curriculum vitae and thus securing employment opportunities after graduation. However, the link between the internship and their long-term career planning is not always as clear as daylight. They seldom have a complete picture of their capabilities, personalities and ambition beforehand, perhaps not even a distinct career path.

Interns might simply send out their curriculum vitae to different companies and wait for replies, without fully understanding whether the internship offered actually matches their personal interests. Ultimately, they obtain internships by chance. Inadequate understanding of the company and the job blemishes their internship experience. Once the interns get their offers from their host companies, they typically do not know what to do until or after the first day. They knew what they actually had to do only when ordered by the seniors. This makes them seem like mere helpers in the company and do not really to add value to the executive sections. For example, jobs like filing, monitoring stock trend from the computer screen for the whole day, photocopying etc, are assigned to interns. As a result, their enthusiasm fades over time, along with their curiosity about the job because they commit to only routine stuff day by day.

To summarise, as the saying goes, "Well begun is half done". In general, interns found their internship programme not as enjoyable as expected, mainly because of their lack of preparation prior to the internship. Further, some of the interns were unable to develop a significant understanding of their long-term goals.

The Ideal Internship and Action Phase

So far the picture looks quite grim, you might not have a good internship experience and it's all YOUR fault. However let us nurse your wounds by taking a glimpse into the soothing concept of a 'dreamy' and 'ideal' internship and how you can take simple yet effective steps to live your dream. **Disclaimer:** we are still students and constantly experimenting with our own and others' philosophies, so don't treat our suggestions as an infallible get employed-quick scheme. Now step into dreamland right now and fantasize about your ideal internship. What would make it ideal? If your boss wants to take you on full-time? Free food and beer? Several pats on your

back for doing a good job every day? Networks with some corporate hotshots to look out for you whenever you need them? Well whatever it may be, the truth is, it's highly subjective and varies greatly from person to person. From the scope of our understanding, interns mostly prioritize factors such as effective networking, challenging tasks, having consistent feedback from the boss and being considered as part of the company. These 'ideal' internship factors are not exorbitant by any means and are definitely achievable.

A simple first step towards obtaining your 'ideal' internship is to write a personal career plan. A well-defined career plan consists of career interests (e.g. Pharmaceutical Industries), short-term goals (3 years), long-term goals (5 - 10 years) and most importantly, an action plan. An action plan, as the name suggests, is a set of activities to do or steps you follow to achieve your career-related goals. For example, if your career interests lie within the automotive industry and your goal is to get an internship at an automotive firm, the first action item could be simply to just shortlist automotive firms you wish to work for. It is crucial that your action items are realistic and ones that can be implemented NOW. Remember those are your stepping stones to your goals; as you tick-off each action item, you can start thinking about more. Continuing on the previous example, you can possibly invite the representative of the automotive firm to give a talk at your university. By engaging in this process, you are creating a network with the representative and also creating a win-win situation for both the representative and yourself. You are giving the representative and his/her firm recognition and in the process, creating a network with the representative. Besides helping you shortlist relevant internship placements, a career plan also comes in handy after you secure an internship. In order to achieve your 'ideal' internship conditions, your career goals have to be aligned with your internship role. Therefore, you can send your prospective boss your career plan to make him/her aware of your desired objectives which gives your potential boss a good idea of what roles you seek in their company. It also establishes a clear communication channel with your boss prior to the internship which will definitely be of great importance when your internship begins. Establishing contact with your boss will also help you receive invaluable feedback on your career or life in general and help you understand him better which will save you time lost in adjusting to a new environment when the internship begins; so you can hit the ground running.

When it comes to networking, normally university students would be too shy to even approach the person or at best, muster the courage to blabber some irrelevant self-description and exchange contacts with each other. This process is too passive and does not add value to anyone, not you and definitely not the person you wish to create a network with. So how would you create a network despite having virtually nothing to offer? One way to go about this issue is to flatter them by asking them about their road to success or some career advice to a prospective graduate such as you and for example, you could even document their successful life story in a simple university newsletter. It's hard to say no to someone who wants to praise you. By doing this, you have again created a win-win situation and hopefully secured an invaluable contact for future use.

On a similar note, developing your digital persona on LinkedIn is also a very powerful tool for both searching for internships and for remaining in touch with your networks. Recruiters, both big and small, are on LinkedIn, so there's no reason you shouldn't be. Obviously because recruiters are strapped for time, your profile summary has to shine and be convincing on first glance. For university students, a good summary would consist of; basic degree information such as the duration, qualification and achievements which can be measured tangibly. For example, instead of writing that you won a design competition, it will be more appropriate to state that you were selected amongst 26 teams in a global design competition. This leaves a more powerful impact on the potential recruiter or any other person for that matter. The summary can also include things you do externally such as volunteering or association activities etc. If you do too many external activities, prioritize the ones you believe are important. As an ending note, you should mention your specialities or strong attributes such as language fluency, leadership, risk management etc. But then again, don't only do a good summary and leave your profile blank, take some time out to develop a holistic LinkedIn profile to account for further interest from people.

In summary, some key takeaways to have a successful internship:

- **Your profile - Create a competent digital profile (Such as LinkedIn)**
- **Your needs - Create a personal career plan**
- **Knowledge - Understand the role and expected duties PRIOR to the internship and it needs to match your career plan**
- **Network - Identify valuable networks linked with your career plan**

In our case, we had a LinkedIn profile prior to our internship. This enabled us to research more about our prospective supervisor and understand his role in the company. We communicated our needs to our supervisor by creating a career plan. Subsequently, we hosted a teleconference with him and voiced our expectations regarding the incoming internship. This also enabled us to gauge our situation prior to the internship, can he offer what we really want, will we be in close contact with him and so on. It is of utmost importance that the roles and expected duties of your incoming internship matches with your needs as stated in your career plan. With regard to networking, we attended a conference wherein we identified our potential targets (obviously in line with our career plan) and interviewed them for an article to be represented in a leading journal.

We really enjoyed our internship and found it valuable. We wish all the interns can obtain the right knowledge and network during this valuable time.

Signing off after a great internship experience

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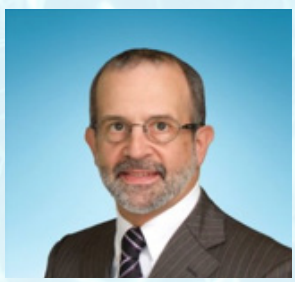
What's next in store for the healthcare industry?

In the modern day business milieu, it is evident the growth of new products are reaching new speeds, with countless innovations and inventions bombarding society at an overwhelming rate. The same trend is evident in the healthcare industry. According to a survey conducted by McKinsey Global, sales of medical devices are growing consistently at a staggering 9% annually, a clear indication of the potential growth and high demand in the industry. However, the downside to this rapid growth rate is the increasing difficulty in obtaining the competitive edge for healthcare companies, given the existing oligopolistic nature of the healthcare industry. The same survey showed that 61% of the 2240 executives interviewed felt skeptical of their company's ability to introduce an innovative product in the market. So amidst all this stiff competition, how does a company ensure success in the future?

Whilst in search of some answers, I was fortunate to meet and have a brief discussion with the healthcare industry's leading expert on patient innovation policy, Mr. Byron Sigel. Mr. Sigel has enjoyed a distinguished career both inside and outside the healthcare industry. He started his career in the U.S. government as a diplomat and trade negotiator focused on Japan. . In the private sector, Mr. Sigel has held the positions of Vice President, Government Affairs at Goldman Sachs in Japan, and Senior Director for Corporate Affairs at Eli Lilly. Mr. Sigel received his Ph.D. from the University of Tokyo. He currently serves as Senior Director of Patient Innovation Policy in Japan for Baxalta, a leading multinational bioscience and pharmaceutical company. Excerpts from the 40-minute interview follow:



R. Byron Sigel
A leading expert on policy making and innovation.
He recently spoke to the Asia Healthcare Journal and emphasized on the importance of human capital to ensure a sustainable future for the healthcare industry and believes that a rise in personalized healthcare for patients is likely.

Could you tell us a bit about your successful life in the healthcare industry and some of your motivating factors to join the industry?

There are multiple reasons as to why I joined the healthcare industry. I was always been interested in policy. I was a Deputy Assistant Trade Representative at USTR, and am now a Vice Chair of the Healthcare Committee for the American Chamber of Commerce in Japan and of course my current role in Baxalta. So, it's definitely a role that I'm very comfortable with. Success in policy making combines several different factors. I think of it as ecosystem that brings together the fields of science and technology, business and public opinion. When you can bring all these factors in unison it's possible to develop an effective policy whose primary objective is to ensure better outcomes for patients. I believe that my success as a policy maker comes in part from a clear understanding of patient needs and their perspective. Good healthcare companies always hold a very high regard for patient needs and opinions. In addition, my training as a scientist and my years of experience on the business side in the private sector I think has enabled me to make better-informed and comprehensive policy recommendations and decisions.

In addition to having good 'know-how' of the industry, good people skills and self-development are also important. I believe that my exposure in various different industries even outside healthcare, has definitely given me a wide range of 'marketable' skills. The variety of my experiences I believe has enhanced my policy making ability as I am able to evaluate different stakeholders' interests whether from government or business based on personal experience. On the lighter side, I believe that I have a pretty good sense of humor and am easily approachable. Many people get intimidated by job titles, so I work hard to put people at ease. Being a good listener is also a trait I value deeply. It is important to engage people in a conversation not just press ones point of view. I learn a lot from the people I interact with. Humour is especially important, it keeps the stress at bay.

In Japan, the healthcare system is primarily regulated by the government, what are the main roles you undertake and what does the future look like for the industry in such countries?

Government affairs often is viewed as a support function in business. I don't see it that way at all. My job is to shape the external environment in ways that create a legal and regulatory framework supportive of innovation. Such an external environment will, I believe, be of direct benefit to our business performance. I do this by establishing dialogue with a wide range of policy stakeholders, including government officials, academics, other industry representatives and patient groups. In addition, I also am responsible for the pricing and reimbursement of our products under Japan's national health insurance system.

Regarding the future of healthcare companies in countries such as Japan, there are still a lot of opportunities. Many healthcare companies have enjoyed great stability and growth in Japan. It is home to premium healthcare services and facilities and will continue to be in the future. A couple of the greatest benefits for healthcare companies in Japan is the availability of highly talented people and its great infrastructure to facilitate innovation and development. Being a technological hub, people have a good 'know-how' of R&D skills and believe in the importance of continuous improvement and innovation. If companies continue to effectively use skilled human resources and modern infrastructure in unison, it is definitely a sustainable recipe for success today and in the future.

What is your take on the future of healthcare for patients? For example, do you see the introduction of wearable health technology a possibility in countries such as Japan?

Arise in personalized healthcare for patients is likely. I won't be surprised if we start seeing remote tracking of dialysis patients in their homes, cardiac arrest predictors and other similar ITC-based applications; the scope is endless. Personally, I think wearables might have a big future in Japan. People here love gizmos and health-related electronics. Wearable health technology will be regarded as a huge evolution in the healthcare industry by by-passing traditional health care delivery through hospitals, clinics, regular doctor visits etc. It is a great way of empowering the patient, the personalized information from the device as real-time feedback keeps them up to date at any given instant thus eliminating the uncertainty of possible health-related changes in their condition they might not be aware of. In terms of stakeholder interests, it ticks most of the boxes in the 'ideal' product checklist. The government has limited resources so the introduction of personalized systems could potentially provide them a platform to better optimize their investment and focus more on higher risk diseases. Regarding doctors and medical institutions, wearables will provide them remote access to patient data and trends. This will help them identify high risk patients thus creating a win-win situation for both the doctor and the patient. Simple mistakes such as incorrect dosage can be avoided with mobile patient data monitoring. Wearable technology can also act as an effective data platform for enhanced R&D purposes as real-time and accurate data can be obtained.

Having said that, I believe it is important to consider the flipside of wearable health technology. My biggest concern is patient data protection. Wearables, like any other IT device, can be susceptible to hacking and cyber-crime. Hackers can use this data against people and can engage in malpractices such as illegal selling of patient data. Another significant issue with wearables is its accuracy. These devices must work perfectly as people's lives are at stake. Any false reading can have serious implications for the patient. Furthermore, the introduction of wearable technology will demand new structures and infrastructure-remodelling. For example, hospitals will have to be equipped with data centres and warning systems in the event that a serious incident has occurred to a patient or could potentially occur. This likely will result in substantial costs for the government and the healthcare institutions. From an industry perspective, another issue for policy makers is where to draw the line between what makes a wearable an accessory or a medical device.

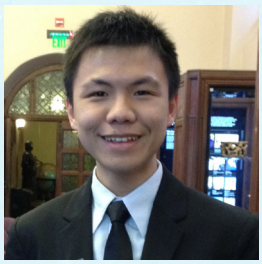
Nevertheless, I do believe that wearables are an exciting innovation and will play a crucial role in healthcare delivery in due course.

Lastly, what would be some key takeaways from 'Sigel's Master Class' for students like me to succeed in the healthcare industry?

I believe that life is a contact sport - you have to get out and do things, meet people, put yourself in challenging environments. And when you fall down, you get up and carry on. Fundamental success factors such as determination, hard-work, and passion are crucial to succeed in the healthcare industry or any industry for that matter. I observe that people who succeed in business have embraced these fundamental success factors into their lives.

I also believe it's critical to find a career where you can achieve alignment between your personal beliefs and your work. . Be adaptable and value the importance of learning and engaging with people. Success is also dependent on people who believe in your potential as much as you do. Also, you can make yourself more 'marketable' by being open to trying out different jobs if they excite you, no matter how disconnected they may seem on the surface. You might just end up thanking yourself for those experiences.

Finally, be sure to nurture your sense of humour, it helps in your human relationship while keeping the stress at bay.



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Behind the Story: Efforts to Be a Medical Regulator – an Interview with Dr. Churn-Shiouh Gau

Accompanying with the emerging ageing problem and increasing demand for advanced medical technology, development of standardised medicinal products has been booming these years. To better administrate the quality of those drug products, countries established their regulatory authorities to manage the registration and licensing work before the drugs ready for sale.

In Taiwan, in order to balance and weigh up the costs and benefits of stakeholders in the industry, a non-government and non-profit organisation named The Center for Drug Evaluation (CDE) was founded in 1998. The organisation works with the Taiwan Food and Drug Administration (TFDA) in performing review of medical products.

Dr. Churn-Shiouh Gau is a veteran medical regulator in Taiwan and in Asia. She started leading the Center for Drug Evaluation (CDE) as Executive Director in 2011. She received her PhD degree in Pharmaceutics from the School of Pharmacy, University of Wisconsin-Madison in the US in 1992, then returned to the School of Pharmacy, National Taiwan University (NTU) as a faculty member. During 1996 to 2000, she was appointed as the Director of the Department of Pharmacy, NTU Hospital, one of the largest university-affiliated medical centres in Taiwan.

Dr. Gau (right) shared her opinions on career planning in regulatory affairs

What is the prerequisite of undertaking the drug evaluation work?

First, you have to gain sufficient knowledge of pharmacy to be capable of doing the assessment work in the pharmaceutics fields. Take my past experience as an example, I was once a teaching assistant when I was pursuing my PhD degree in the US, this granted me opportunities to understand the nature of subjects within the medical field, which is useful for my future career development. For instance, you can be able to evaluate the product functions only when you are familiar with the drug dosage. Besides the fundamental knowledge of medicine, you need to be quick-witted to comprehend the connections between the academic subjects and practical operations. As for regulatory science, you cannot grasp the complete picture of your work and the industrial ecosystem by only studying medical theories, you have to be clear-headed to approach cases from diverse aspects. To a large extent, my once being the director of the hospital pharmacy provided me with exposure to the hands-on management skills.

Is a background of bioengineering capable of working for drug regulation?

If you have a bioengineering background, it could still be difficult for you to undertake the drug evaluation job. Since the field of bioengineering is more related to the advancement of biotechnology, which is different from the conventional pharmaceutical studies. You may find it easier if you keep on striving for a postgraduate degree in pharmacy and thus developing concrete foundations for medical studies before entering the industry. The qualification requirements actually vary from place to place, I suggest you looking for relevant information from the authorities you are interested in and then planning for your goal.

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What guidelines or principles should always bear in mind when working in your profession?

Our work on drug evaluation acts as a communication platform for the officials and the company applicants and provides advisory services to those applicants during their registration. Therefore, we have to be familiar with and sensitive to the development of health policy. Further, our work helps strengthening the quality and efficiency of drug review, and most importantly, looking after the safety concerns of the end-users. In general, it is all about public health and regulations. Decisions made by our section have great impacts on the industry. Hence, we have to be attentive when scrutinising every piece of applications. Also, balancing points of view is essential during assessing costs and benefits of stakeholders. We can then formulate the regulatory framework in the right direction eventually.

If I want to be a medical regulator like you, what would you advise me to plan and prepare from now on as an undergraduate?

Medical regulator assists the technical evaluation for market approval of drugs and medical devices. If you want to be a medical regulator, it would be better for you to study in graduate school for a master or a PhD degree. You need to consolidate your knowledge of biology and pharmacy before undertaking the job. Besides, having the competence to communicate effectively and efficiently with different parties is important. It requires you to have adequate understanding of the actual operations in the industry. Especially when examining the product development, you should have a clear picture in your mind of the procedures involved. Before gathering information to work on regulation, you need to know and sort out the connections between different parts in essence. For your better accumulating knowledge of the industry, you may wish to take part in internship programmes in the pharmaceutical industry or in other related fields. Also, it would be helpful for you to seek advice and recommendations from your university professors, so as to build up your desired career path.

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