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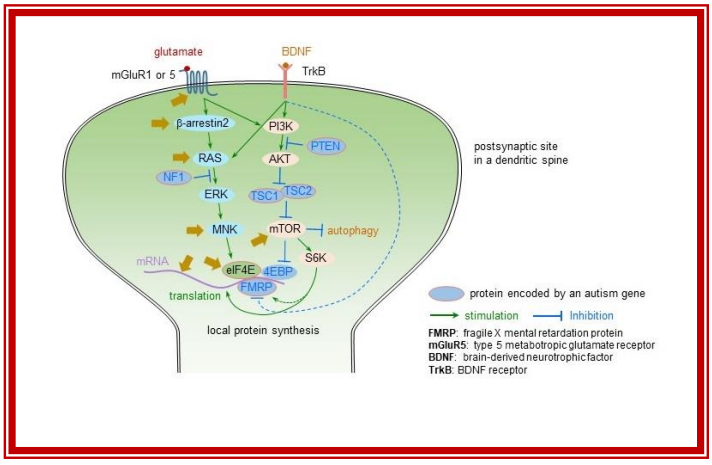

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Original Research

Review

Case Report

Original Research

Introduction of a New Video-Based Eye Tracking Paradigm for Early Detection of ASD

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Recently, there is growing interest inapplying eye tracking technology to study infants and young children with Autism Spectrum Disorder (ASD). As a non-invasive and convenient measurement, it uses relatively objective parameters, which will greatly avoid the possibility of bias in traditional subjective evaluations caused by asymmetric information between patients (or parents) and examiners. As a result, it has been considered as having the greatest direct clinical potential for early screening for ASD. This study aims to introduce a new video-based eye tracking paradigm. The paradigm consists of 10 video scenarios, with each scenario targeting a different aspect of ASD in infants and children. The total paradigm lasts about 2 minutes. We believe this eye tracking paradigm may be a useful tool for early screening for ASD.
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Key Words: *autism spectrum disorder, eye tracking, stimulation paradigm, facial movement, facial gestures, gaze*

INTRODUCTION

Autism spectrum disorder (ASD) is a group of complex neurodevelopmental disorders with a rapidly rising rate of incidence. The etiology and pathophysiology of ASD are not well understood, and there is currently no effective treatment or cure. More than 70% of individuals with ASD need lifetime care, and the Centers for Disease Control and Prevention (CDC) has called it a national healthcare crisis.

A growing body of evidence suggests early diagnosis and intervention can significantly impact the prognosis of individuals with ASD. The earlier the detection and diagnosis, the better the prognosis and functional status later in life. The current average age for diagnosis is around 4 years of age, but ASD individuals show signs as early as infancy. Development of an easily-applied early detection tool and screening test is crucial and has drawn the attention of investigators in recent years.[1]

The fourth and fifth editions of the Diagnostic and Statistical Manual of Mental Disorders and the tenth edition of the International Classification of Diseases emphasize that an early onset of symptoms is essential for core autism and other forms of ASD.[2] Nevertheless, the majority of ASD studies have been carried out on subjects past mid-childhood,[2] which have significantly impeded the development of early diagnosis and intervention methods.

Currently, the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview - Revised (ADI-R) are considered the 'gold standard' in diagnostic evaluations for autism, but both methods can only be performed by trained professionals, usually require a considerable waiting time for both the test and the report, are not applied on children younger than one year old, and may be subjective and variable. Overall, there is a shortage of resources for early evaluation. As a result, most children with ASD are diagnosed in later childhood. To improve this situation, easier and faster alternative methods, especially for younger children, are urgently needed.[2-3]

Generally speaking, the tools currently applied to explore ASD can be classified into two categories: subjective tools such as questionnaires, observation scales, interviews and developmental tests and objective measurements such as eye tracking and brain imaging tools, including electroencephalography (EEG), event-related potentials (ERPs), magnetoencephalography (MEG), functional and structural magnetic resonance imaging (MRI), diffusion tensor imaging (DTI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), and

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near infrared spectroscopy (NIRS). Each of these tools have pros and cons. Subjective tools are widely used in clinics and are still the predominant method for diagnosing and evaluating autism, but they are hampered by subjective bias. Although objective tools can provide objective measurements and hold great potential, their connection with clinical symptoms remains undetermined.[2-3] Also, though accumulating evidence has demonstrated that ASD is associated with brain morphometry and functional changes as compared with typically developing (TD) controls,[3-4] the high expense and complexity of data analysis and interpretation have significantly limited the application of brain imaging tools such as MRI, PET, DTI and MEG.

One objective tool that is promising and is being increasingly applied in ASD clinics is eye tracking (ET).[5] As a non-invasive and convenient measurement, it uses relatively objective parameters, which will greatly avoid the possibility of bias in traditional subjective evaluations caused by asymmetric information between patients (or parents) and examiners.

Eye tracking can measure the point of gaze (where one is looking) and the motion of the eyes relative to the head. Eye movements and pupillary motility are tightly regulated by brain circuits and indirectly reflect functional and structural changes in the brain. Thus, exploring how children use their eyes in various contexts may reflect their learning and development processes in the brain.[5] As a result, there is substantial growing interest in eye-tracking, particularly when studying infants and young children. It is considered to have the highest direct clinical potential for early screening for Autistic Spectrum Disorder.[2]

Currently, various stimulus paradigms have been developed in eye tracking studies. These paradigms have predominantly used dynamic social scenes.[3,5-9] Although these visual paradigms can distinguish ASD children from typically developing controls, they tend to be too complicated for young infants and thus are of limited use.

This manuscript aims to develop a short and simple paradigm that can be applied for infants, toddlers and children to catch early signs of ASD in eye gaze, eye following, joint attention, and emotion response. This paradigm can be used to detect early, subtle and unique cues for infants at risk to ASD. In addition, although this paradigm is designed for an Asian population (by using a Asian actress), it can be easily optimized to other populations due to its simplicity. The paradigm consists of 10 scenarios, and the total test lasts around two minutes.

Rationales for an eye tracking paradigm

Eye contact plays an important role in social interaction. Facial movements, gestures, and direction of one's gaze provide critical information about a person's intentions and emotions. Gaze direction and duration provide information about what the other person is interested in.[5,8] Thus, this paradigm includes several scenarios, including a woman talking (without sound), a woman talking presented alongside a white dot moving in a circle, a sad face next to a neutral face, a happy face next to a neutral face, and a face with the eyes looking side-to-side. In addition, we also have a photograph of an infant's face presented next to a picture of a fan.

Preferential attention to biological motion represents a basic mechanism in humans and monkeys facilitating adaptive interaction with other living beings.[10] Investigators have found that two-year-old infants with ASD fail to orient towards point light displays of biological motion, and their viewing behavior when watching these point light displays can be explained as a response to non-social, physical contingencies - physical contingencies that are different from control children.[10] Specifically, ASD children attended less to upright biological motion than did TD toddlers. The findings were further replicated by other studies.[9,11] Thus, in this paradigm, we include video of a point light display of a human walking next to an inverted version of the figure (i.e., upside-down biological motion). In addition, we also show a clipart duck moving horizontally from the left side of the screen to the right side followed by a clipart helicopter moving vertically from the top of the screen to the bottom, to further explore how children watch non-human motion.

Circumscribed interest refers to a type of repetitive behavior frequently observed in children with ASD. It is characterized by intense interest in a narrow range of subjects and by rigid organization of activities exclusively around this interest.[12] We thus also present an array of multiple items including both high-autism-interest (HAI) objects such as vehicles and computers, as well as low-autism-interest (LAI) objects such as furniture or clothing to investigate the circumscribed interest of children.

Each video will contain one or more Areas of Interest (AoI) covering the parts of the screen that may potentially be used to distinguish between ASD and TD children. We will measure the duration of the point of gaze in each AoI, as well as the duration of gaze outside any AoI. For example, in the video of the speaking woman, the woman's eyes would constitute one AoI and her mouth would constitute another, to see if the child favors looking at the eyes, the mouth, or neither. In the point light display video, we can use AoIs covering each figure to see which figure is viewed longer or more often.

Detailed description of the eye tracking paradigm

Scenario 1a (5 seconds): The video will show a woman sitting still and looking directly at the camera. One AoI will cover the eyes, and the other AoI will cover the mouth.

Scenario 1b (5 seconds): The video will be similar to Scenario 1a, but the woman will be mouthing the alphabet. One AoI will cover the eyes, and the other AoI will cover the mouth.

Scenario 2a (5 seconds): A point light display figure of a person walking upright will be shown on one side of the screen. On the other side, the same figure will be shown rotated 180 degrees, with the person appearing to walk upside down. Each figure will be an AoI.

Scenario 2b (5 seconds): This is identical to Scenario 2a, but with the positions of the figures switched (left vs. right side of the screen).

Scenario 3a (5 seconds): A video of a dot moving along a circular path will be displayed on one side of the screen, while a video of a woman mouthing the alphabet will be shown on the other side. Each video will be an AoI.

Scenario 3b (5 seconds): This will be identical to Scenario 3a, but with the positions of the videos switched.

Scenario 4 (10 seconds): A clipart duck will be shown moving horizontally from the left side of the screen to the right side. This will be followed by a clipart helicopter moving vertically from the top of the screen to the bottom. The two images will be the AoIs.

Scenario 5a (5 seconds): An image of a fan will be presented next an image of an infant's face. Each image will be an AoI.

Scenario 5b (5 seconds): This will be identical to Scenario 5a, but with the positions of the images switched.

Scenario 6 (10 seconds): An array of objects will be presented on the screen. Roughly half of the objects will be high-autism-interest (trains, planes, electronics, etc.), and the remaining objects will be low-autism-interest (clothes, furniture, food, etc.). Each object will be an AoI.

Scenario 7 (25 seconds): A woman holding a tablet will be shown on the screen. She will look directly at the viewer for a few seconds, then turn on the tablet. On the tablet, various moving, colorful shapes will be displayed. After a few seconds, she will turn off the tablet and look back at the viewer. The AoIs will cover the woman's face and the tablet.

Scenario 8a (10 seconds): An isolated face of a woman will be displayed in the center of the screen. After a few seconds of looking directly at the viewer, the woman will look to one side. The AoI will be the woman's eyes.

Scenario 8b (10 seconds): This will be similar to Scenario 8a, except that the woman will look to the other side.

Scenario 9a (5 seconds): Two videos of a woman, one with a sad face and one with a neutral face, will be presented side by side. Each face will be an AoI.

Scenario 9b (5 seconds): This will be identical to Scenario 9a, but the positions of the videos will be switched.

Scenario 10a (5 seconds): This will be identical to Scenario 9, but with a happy face and a neutral face.

Scenario 10b (5 seconds): This will be identical to Scenario 10a, but the positions of the videos will be switched.

We believe this paradigm will provide crucial information on different aspects of ASD in children, making it a useful tool for early screening. The next step would be to collect real data to test how well the paradigm can distinguish ASD children from TD children.

CONFLICT OF INTEREST

JK has a disclosure to report (holding equity in a startup company (MNT)), but declares no conflict of interest.

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AUTHOR CONTRIBUTIONS

Xuejun Kong and Jian Kong have equal contributions for conceiving the ideas, developing the protocol/paradigm and organizing the project.

Bryan K. Wang, helped to write the first draft, participated the project, searched and organized references, finished English proof reading.

Joseph Park helped the video production, technical support, editing and proof reading.

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Original Research

Study on the Chemical Components of the Ethyl Acetate Extract from *Herpetospermum Caudigerum*

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***Herpetospermum* seed, a common folk medicine used by Tibetan medication, is the dried ripe seed of *Herpetospermum Caudigerum* Wall. It is bitter in taste and cold in nature. In Tibet it is popularly known and used in traditional medicine for the treatment of liver diseases, cholic diseases, and dyspepsia. Six compounds, named Herpetin(1), Eicosanoic acid, 2-propenyl ester(2), Cucurbitacin R(3), Cucurbitacin L(4), 3'-Hydroxydaidzein(5), Oleanic acid(6), have been isolated from the ethyl acetate extract of the seeds of *Herpetospermum Caudigerum* Wall, among these compounds, compound 2, 3, 4, 5 were isolated from this plant for the first time.**

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Key Words: *herpetospermum caudigerum wall, cucurbitacin, extraction, isolation*

INTRODUCTION

Herpetospermum Caudigerum Wall (Cucurbitaceae) is distributed in southwest China. The dried ripe seeds of *Herpetospermum Caudigerum* Wall. have been used for the treatment of liver diseases as a Tibetan folk medicine in China.[1-3] The study crushing the dried seeds, then extracting the chemical composition by 95% ethanol. Collecting eluate, evaporating solvent, the concentrated solution dispersed in water, then obtainning different fractions by extracting with petroleum ether, ethyl acetate and *n*-BuOH.[4] The sugar and grease in the ethyl acetate extract was removed. Then repeated using the technical means include Silica gel column chromatography, Sephadex chromatography, reversed-phase column chromatography, recrystallization and semi-preparative column chromatography to separate and purificate until obtainning the monomer compound.[5] Finally, the compound structure was identified by TLC, ^{1}H-NMR, ^{13}C-NMR, ect. We identified the structures of 6 compounds (**Figure 1**): Herpetin(**1**), Eicosanoic acid, 2-propenyl ester(**2**), Cucurbitacin R(**3**), Cucurbitacin L(**4**), 3'-Hydroxydaidzein(**5**), Oleanic acid(**6**), among this compounds, compound **2-5** were first isolated from *Herpetospermum* seed.

METHODS

General Experimental Procedures

1D and 2D NMR spectra were taken on a BRUKER AVANCE III NMR System-600 NMR spectrometer. ESI-MS and HRESIMS were obtained using an Agilent 6210 TOF LC-MS mass spectrometer. Preparative HPLC was performed on an Agilent 1260, and a reversed-phase C_{18} column (YMC-Pack ODS-AU 20×250mm, 10μm) was employed. Column chromatography was undertaken over silica gel (200-300m).[11] TLC was carried out with glass plate precoated silica gel G. Spots were visualized under UV light and by spraying with 10% H_2SO_4 in 95% EtOH, followed by heating at 100ºC. Methanol used in preparative HPLC procedure was in HPLC grade, and other solvents were of analytical grade.

Plant Material

The seeds of *Herpetospermum Caudigerum* Wall. were collected from the kangding area in Sichuan province and authenticated by A/Prof. Liang-Ke Song in School of Life Science and Engineering, Southwest Jiaotong University. Avoucher specimen was deposited in Room3704, 3rd Teaching Building, Southwest Jiaotong University.

Extraction and Isolation

The dried-up and powdered seeds of *Herpetospermum Caudigerum* Wall. (10 kg) were extracted with 95% EtOH at room temperature. After removal of the solvent, the residue after removing EtOH was suspended in water and partitioned with petroleum ether, ethyl acetate, *n*-BuOH, successively.

The ethyl acetate extract (280g) was subjected to column chromatography on silica gel with gradient solvents of DCM-MeOH (100:0-1:1). The collected fractions were combined according to the TLC result to give 9 fractions (BL_1-BL_9). BL_4 (46g) was applies to silica gel column chromatography to give $BL_{4\text{-}1}$ (10.4g), $BL_{4\text{-}1}$ was purified by silica gel column chromatography eluted with $CHCl_3$:MeOH (100:3-100:10)

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and the use of Sephadex LH-20 (CH_3OH) to yield compound 1 (18.9mg) and compound 2 (20.1mg). According to the TLC profiles, BL_{4-7} (9.6g) was applies to Silica gel column chromatography eluted with petroleum ether:acetone (50:1~0:1) to give BL_{4-7-1} (2.3g), BL_{4-7-1} was purified by the use of Sephadex LH-20 (CH_3OH) to yield compound 3 (30.2mg). Then BL_{4-7-5} (3.1g) was applies to silica gel column chromatography to give $BL_{4-7-5-1}$ (203mg), $BL_{4-7-5-1}$ was purified by use of silica gel column chromatography, reversed-phase column chromatography and Sephadex LH-20 (CH_3OH) to yield compound 4 (9.8mg). $BL_{4-7-5-4}$ (96mg) was applied to preparative HPLC system [mobile phase: CH_3OH/H_2O (65:35, v/v); flow rate: 5 mL min^{-1}; UV detection at 254 nm] resulting in the isolation of compound 5 (14.8mg). BL_{4-8} (7.8g) was applies to silica gel column chromatography eluted with petroleum ether:ethyl acetate (30:1~0:1) to give BL_{4-8-1}, BL_{4-8-1} was purified by the use of Sephadex LH-20 (CH_3OH) to yield compound **6** (1.02g).

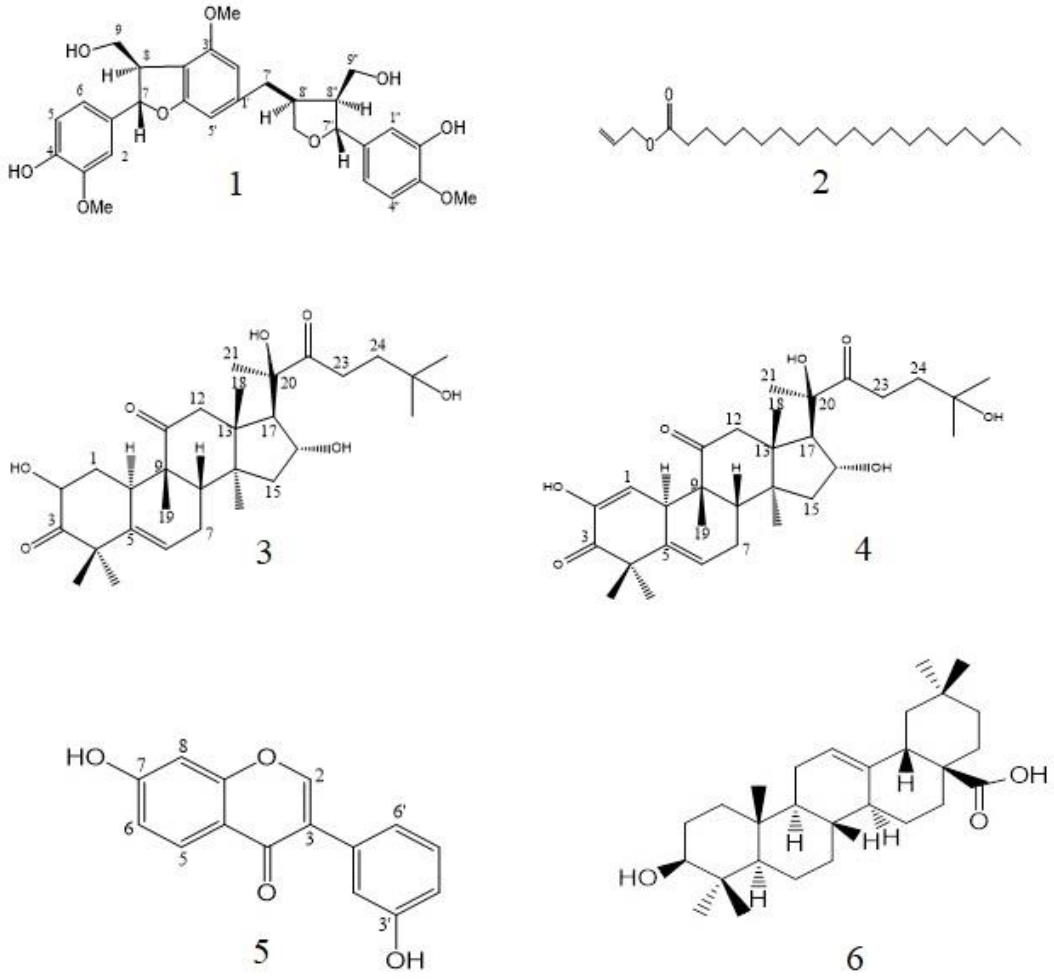

Figure 1. The structures of compounds 1-6.

RESULTS AND DISCUSSION

Compound **1** was obtained as a white amorphous powder, and determined to possess the molecular formula $C_{30}H_{34}O_9$ by its pseudo-molecular ion peak at m/z 561.2160 $[M+Na]^+$ in the positive HR-ESI-MS experiment. According to the data of ^{1}H-NMR (CD_3OD, 400MHz) δ: 6.94 (1H, d, J =2.0Hz, H-2), 6.90 (1H, s, H-1"), 6.90 (1H, s, H-4"), 6.88 (2H, d, J = 8.0Hz, H-6, 5"), 6.82 (1H, d, H-6), 6.68 (1H, s, H-2'), 6.67 (1H, s, -OH), 5.54 (2H, d, J = 6.4Hz, -OH), 4.77 (1H, d, J =7.2Hz, H-7"), 4.08 (1H, m, -OH), 3.93 (2H, m, H-9), 3.89 (3H, s, $-OCH_3$), 3.88 (3H, s, $-OCH_3$), 3.86 (3H, s, $-OCH_3$), 3.77 (2H, m, H-9"), 3.61 (1H, m, H-8), 2.94 (2H, m, H-9'), 2.74 (2H, m, H-7'), 2.58 (2H, m, H-8', 8"), 2.42 (2H, m, H-7). Its structure was identified as herpetin [6,7] by comparison of the spectrum of data with those reported in the literatures.

Compound **2** was obtained as a white granular crystal, and determined to possess the molecular formula $C_{23}H_{44}O_2$ in the positive HR-ESI-MS experiment. According to the data of ^{1}H-NMR (400 MHz, $CDCl_3$): δ5.32-5.17 (2H, m), 4.22 (1H, dd), 4.07 (1H, dd), 2.26-2.22 (3H, m), 1.99-1.91(4H, m), 1.20 (30H,

q), 0.81 (3H, t). The structures of compound **2** is simple, and its structure was identified as Eicosanoic acid, 2-propenyl ester [8] by comparison of the spectrum of data with those reported in the literatures.

Compound **3** was obtained as a white crystal powder, and determined to possess the molecular formula $C_{30}H_{46}O_7$ in the positive HR-ESI-MS experiment. According to the data of ^{1}H-NMR (400 MHz, $CDCl_3$): δ 4.38 (1H, s, H-6), 4.30 (1H, t, J = 7.5Hz, H-2), 3.90 (2H, s, J = 4.2Hz, -OH), 3.52 (1H, s, -OH), 3.51 (1H, s, -OH), 3.12 (1H, d, J =14.8Hz, H-16), 2.92 (1H, dd, J =16.1,8.1Hz,H-23), 2.74 (2H, d, J = 13.5Hz, H-7, 12), 2.64 (2H, m, J =14.6Hz, H-7, 12), 2.57 (1H, m, J = 6.9Hz, H-10), 2.40 (1H, m, J = 5.2Hz, H-9), 2.23 (1H, m, J = 4.3Hz, H-8), 2.05(2H, s, H-24), 1.82 (4H, dd, J = 13.3, 6.8Hz, H-1, 15), 1.41 (6H, s, -CH_3), 1.33 (6H, s, -CH_3), 1.27 (3H, s, -CH_3), 1.24 (3H, s, -CH_3), 1.21 (3H, s, -CH_3), 1.18 (3H, s, -CH_3). Its structure was identified as Cucurbitacin R [9] by comparison of the spectrum of data with those reported in the literatures.

Compound **4** was obtained as a white crystal powder, and determined to possess the molecular formula $C_{30}H_{44}O_7$ in the positive HR-ESI-MS experiment. According to the data of ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.14 (1H, s, H-1), 4.13 (1H, s, H-6), 4.10(1H, s, -OH), 4.08 (1H, s, -OH), 3.49 (1H, s, -OH), 3.12 (1H, d, H-16), 2.27 (1H, dd, H-10), 1.81 (1H, d, H-17), 1.78 (1H, m, H-8), 1.62 (3H, s, H-7, 12), 1.57 (1H, m, H-23), 1.30 (1H, m, H-24), 1.28 (6H, m, H-7, 12, 15), 1.27 (3H, s, -CH_3), 1.25 (3H, s, -CH_3), 1.12 (3H, s, -CH_3), 1.10 (3H, s, -CH_3), 1.05 (3H, s, -CH_3), 0.98 (3H, s, -CH_3), 0.94 (3H, m, -CH_3), 0.91(3H, s, -CH_3). Its structure was identified as Cucurbitacin L [9] by comparison of the spectrum of data with compound **3** and those reported in the literatures.

Compound **5** was obtained as a white crystal powder, and determined to possess the molecular formula $C_{15}H_{10}O_4$ in the positive HR-ESI-MS experiment. According to the data of ^{1}H NMR (400 MHz, CDCl3): δ 8.08 (1H, s, H-2), 7.93 (1H, d, Ar-H), 7.51 (1H, d, Ar-H), 7.44 (1H, d, Ar-H), 7.41 (1H, d, Ar-H), 7.31 (1H, s, Ar-H), 7.28 (1H, d, Ar-H), 5.53 (1H, s, H-2'), 5.23 (1H, s, -OH), 4.90 (1H, s, -OH). Its structure was identified as 3'-Hydroxydaidzein[10] by comparison of the spectrum of data with those reported in the literatures.

Compound **6** was obtained as a white needle crystal, and determined to possess the molecular formula $C_{30}H_{48}O_3$ in the positive HR-ESI-MS experiment. According to the results that the TLC indicated that compound **6** could show the same color pot as the Oleanic acid in the same position by using chloroform-methanol (15:1) or Cyclohexane-acetone-ethyl acetate as developing solvent, and concentrated sulphuric acid: ethanol (10:1) solution as coloration using silica gel G, its structure was identified as Oleanic acid.

CONCLUSION

The study crushing the dried seeds, then extracting the chemical composition by 95% ethanol. Collecting eluate, evaporating solvent, the concentrated solution dispersed in water, then obtaining different fractions by extracting with petroleum ether, ethyl acetate and *n*-BuOH. The ethyl acetate extract was repeated using the technical means include Silica gel column chromatography, Sephadex chromatography, reversed-phase column chromatography, recrystallization and semi-preparative column chromatography to separate and purificate until obtaining the monomer compound. Finally, the compound structure was identified by TLC, ^{1}H-NMR, ^{13}C-NMR, et al. We identified the structures of 6 compounds, they were Herpetin, Eicosanoic acid, 2-propenyl ester, Cucurbitacin R, Cucurbitacin L, 3'-Hydroxydaidzein, and Oleanic acid. What's more, Eicosanoic acid, 2-propenyl ester, Cucurbitacin R, Cucurbitacin L, and 3'-Hydroxydaidzein were first isolated from *Herpetospermum* seed, it greatly enriched the type of natural products. During the study, the sugar and grease in the ethyl acetate extract was removed to reduce the interference of glycolipids, it also accelerated the speed of monomer purification.

CONFLICT OF INTEREST

None.

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Original Research

The Relationship between Internet Addiction and Internalizing Problems in Overweight/Obese Adolescents: A Moderated Mediation Model

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To explore the relationship of Internet addiction, coping styles, stressful life events and internalizing problems in overweight/obese adolescents, this research surveyed 1438 middle school students among which 245 overweight and obesity (non-clinical cases) were screened based on the body mass index (BMI) percentile criteria of Working Group on Obesity in China (WGOC) by a series of questionnaires and anthropometric indices. The results indicated as follows: (1) Negative coping styles played a partial mediating role in the relationship between Internet addiction and internalizing problems among overweight/obese adolescents, which means Internet addiction had a direct effect on internalizing problems and also indirectly affected internalizing problems through negative coping styles. (2) The mediating effect of negative coping styles was moderated by stressful life events. The effect of negative coping styles on internalizing problems was in positive proportion to internalizing problems. Internet addiction, coping styles, stressful life events and internalizing problems among overweight/obese adolescents constructed a moderated mediating model.

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Key Words: *internet addiction, coping styles, stressful life events, internalizing problems, overweight/obese adolescents*

INTRODUCTION

Overweight and obesity, which are defined as excessive or abnormal fat accumulation, are chronic metabolic disorders caused by the excessive calorie intake over the amount of energy consumption under the influence of genetic and environmental factors.[1] Ever since the 1980s, the incidences of overweight and obesity disorders grow exponentially. Obesity and its related symptoms have been developed into a major public health concern in developed and some developing countries.[2] The average BMI index increases from 28.8% to 36.9% among adult men from 1980 to 2013 worldwide. During the same period this number increases from 29.8% to 38% in women. In 2013, the overweight/obesity rate among 2-19 years old boys and girls in developed countries are 23.8% and 22.6% respectively. In developing countries these numbers also increase from 8.1%, 8.4% to 12.9%, 13.4% for boys and girls, respectively,[3,4] Similarly, the detection rate of overweight and obesity also increase rapidly in China. From 1985 to 2014, the detection rate of overweight/obesity among students aged 7 to 18 continuously increase, with the annual growth rates of 0.27%-0.63% for overweight cases and 0.1%-0.58% for obesity cases. Moreover, the obesity detection annual growth rate from 1985-2014 reaches its peak from 2010-2014.[5] Overweight and obesity bring significant harms to children and adolescence, and they increase the risk of developing chronic diseases later in life. Studies show that around 80% of adolescence with obesity will develop adult obesity.[6] At the same time, there are close relationships between adolescent obesity with multiple types of cardiovascular and metabolic disorders, including hypertension, hyperlipidemia, fatty liver and type II diabetes. The risks of developing these disorders increase with the early onset of obesity and the prolonged course of the disease.[7] Therefore, the long-term harmful effects of obesity on the health status of adolescents could not be overlooked.

Except from the health concerns, overweight and obesity also have adverse effects on the psychological and behavioral development of adolescents. Internalizing problem behavior is

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defined as the negative and unhappy feelings experienced by individuals, which is mainly manifested as anxiety and depression. Compared to externalizing problem behaviors such as aggressiveness and delinquency, internalizing problem behavior are hard to notice and would not cause direct threat to others. However, it will cause long-lasting hidden problem to the mental health of the individuals.[8] Most of the overweight/obese children will not feel confident with their own body weight and shape. Since obesity causes many inconveniences during their daily activities, these kids easily become the subjects of ridicule among their peers. Moreover, obese students have much less opportunities to go on stage to perform during school activities, which will further reduce their self-confidence.[9] Consequently, their social communication and adaption abilities are usually compromised compared to kids with normal body weight. The dual effects of prejudgment from outside world and their self-consciousness often make obese children feel low self-esteemed, isolated, depressed and anxious, causing their mental illness.[10-12] Compared to the adolescents with normal weight, the depression and anxiety are common mental health issues associated with overweight/obese adolescents. For example, through the meta-analysis of longitudinal studies, Manna found that obesity and depression influence each other; the rates of obesity among depressed adolescents increase by 70%. Vice versa, the risk of depression also increases by 40% among obese adolescents.[13] Recent studies have already confirmed the increased frequencies of depressing and anxious mood among overweight/obese adolescents compared to those with normal body weight.[14] Due to the adverse impact of obesity on the mental health of those affected, research on the mechanism of internalizing problem development among overweight/obese adolescents are crucial to further prevent and intervene these issues.

With the vigorous development and widespread of Internet, it has gradually become a necessary tool during the daily activities for adolescents. However, the issue of Internet addiction has also inevitably developed. Internet Addiction (IA) or Internet Addiction Disorder (IAD), also named pathological Internet use (PIU) or Internet overuse (IO), indicates the loss of control over one's Internet tolerance, withdrawal response and desire to use Internet that are caused by the inappropriate use of Internet in a long time period, which impairs ones physiological, mental and social functions.[15] Due to the Internet addiction, some adolescents show a series of problems including indifferent social relationships, narrow social interactions, poor social adaption, disorganized time management, as well as academic and personal developmental hardship.[16] Studies have shown that pathological Internet usage will cause a number of social relationship and mental problems and will cause mental illness in adolescents, especially introvert boys.[17] Adolescents have become the susceptible group to Internet addiction.[18] Past studies have reached similar conclusions that overuse of Internet has close relations with adolescent mental disorders, social anxiety, depression and suicide.[19] For example, the prospective study conducted by Ko et al[20] has confirmed that the pathological usage of Internet could predict the occurrence of depression and social interaction disorders during the follow-up studies within the next two years. A 9-month follow-up study conducted by Lawrence[21] showed that the risk of developing depression among mentally healthy adolescents with pathological Internet usage at the start of the study is 2.5 times higher compared to those with normal Internet usage. At the same time, other studies show that overuse of Internet among adolescents will exacerbate their existing overweight problems.[22] Moreover, as Internet addiction leads to a more sedentary lifestyle, it may become an independent risk factor to predict the occurrence of adolescent obesity.[23] Therefore, the first task of our work is to study the impact of Interment addiction on the internalizing problems (depression, anxiety) of adolescents.

In this study, another important question to ask is how Internet addiction actually influences the development of internalizing problems among overweight/obese adolescents, and whether Internet addition is an indirect influence that requires mediators. Coping style, also known as coping strategy or coping mechanism, indicates the cognitive and behavioral approaches individuals take when facing stress and challenges. It is an important factor when evaluating the mental healthiness of adolescents.[24] As a factor of internal self-regulation, coping style also has important effects on the development of Internet addiction.[25,26] Basing on the existing literature, we hypothesize that coping style may be an important mediator between Internet addiction and internalizing problems in overweight/obese adolescents. Previous studies show that coping style is related to negative emotions, positive coping style is in negative relation with depression and anxiety, while negative coping style is positively related to these negative emotions.[27] The social cognitive theory indicates that the use of Internet is a social cognitive process, and how individuals use the Internet may reflect their self-regulation capacities. On the other hand, Davis proposed a cognition-behavior model, which indicates the non-adaptive cognition as the proximal and sufficient reason for Internet addiction.[28] Both of these theories emphasized that Internet addiction is related to the cognitive characteristics of the Internet users.[29] Empirical research also supports this point, which shows that individuals who are addicted to Internet exhibit significant differences in terms of self-blame, hallucination, evacuation, rationalization and total negative coping scores. In addition, when facing challenges and stressful events, adolescents who are addicted to Internet randomly try to change the stressful environment through directly solving the problems and asking for help. Most of them negatively cope with stressful events through evacuation, self-blame and hallucination.[30] However, whether coping style could mediate the effects of Internet addiction on overweight/obese adolescents remains unclear. Thus, basing on related theory and research evidence, the second aim of our study is to investigate whether the coping style is a mediator in the process of Internet addiction affecting the internalizing problems of obese/overweight adolescents.

Stressful life events indicate the major events encountered by individuals during their daily life that could acutely and

strongly affect their mental states. They are prone to induce negative psychological responses, which through changing the functions of neurological and endocrine systems, result in physiological and psychological disorders and negatively affect mental health of individuals.[31] Additionally, stressful life events are the inducing factors of depression and anxiety, functioning as 'trigger' during the generation of internalizing problems.[32] The Stress-coping model proposed by Wagner[33] is the most popular explanation for the cognition-behavior theory during the addictive process. Wagner indicates that addictive behavior can be viewed as a coping strategy used by the addicting people in response to stressful events. It could either help relieve the stress brought by the negative emotions, or increase the positive emotions of the addicting people. Accordingly, we predict that different levels of stressful life events will affect the corresponding coping styles. Stressful events could therefore be the moderator of adolescent internalizing problems, and directly exert its effect through affecting the individual's coping style. How do stressful life events moderate the mediating effects of coping styles? Under what kind of circumstances, and how would stressful life events exert their moderating effect? Notably, a pulling force (temptation felt by the Internet users) and a pushing force (stressful life events) coexist during the process of Internet addiction. One needs two independent mental strategies to cope this dual effect. With the stressful life events, 'stress-coping system' is required to eliminate or reduce the negative impacts; while 'temptation-coping system' is needed to prevent one from Internet addiction.[26] It is likely that stressful life events would lead individual to cope through immature behaviors (i.e. hallucination, evacuation, self-blame etc.), in order to reduce the negative emotion and impacts from real-life. When there are a number of stressful life events at the same time, it is more likely that an individual will take the negative coping style, which facilitates the generation of internalizing problems. When the number of stressful events decreases, the predicting effects of negative coping behaviors on internalizing problems decrease. This shows that stressful life events may play a moderating role in the second half of the mediating chain of coping style, i.e. positive moderating effect between the indirect relation of Internet addiction and internalizing problems.

In summary, the major goal of this study is to discuss the effects of Internet addiction, coping style and stressful life events on the internalizing problems in overweight/obese adolescents, and to study the interaction mechanisms of these factors. Basing on the previous studies, we hypothesize that coping style plays a mediating role between Internet addiction and the internalizing problems in overweight/obese adolescents. This mediating effect is positively regulated by stressful life events. Internet addiction has moderated mediating effect on the internalizing problems in overweight/obese adolescents (**Figure 1**).

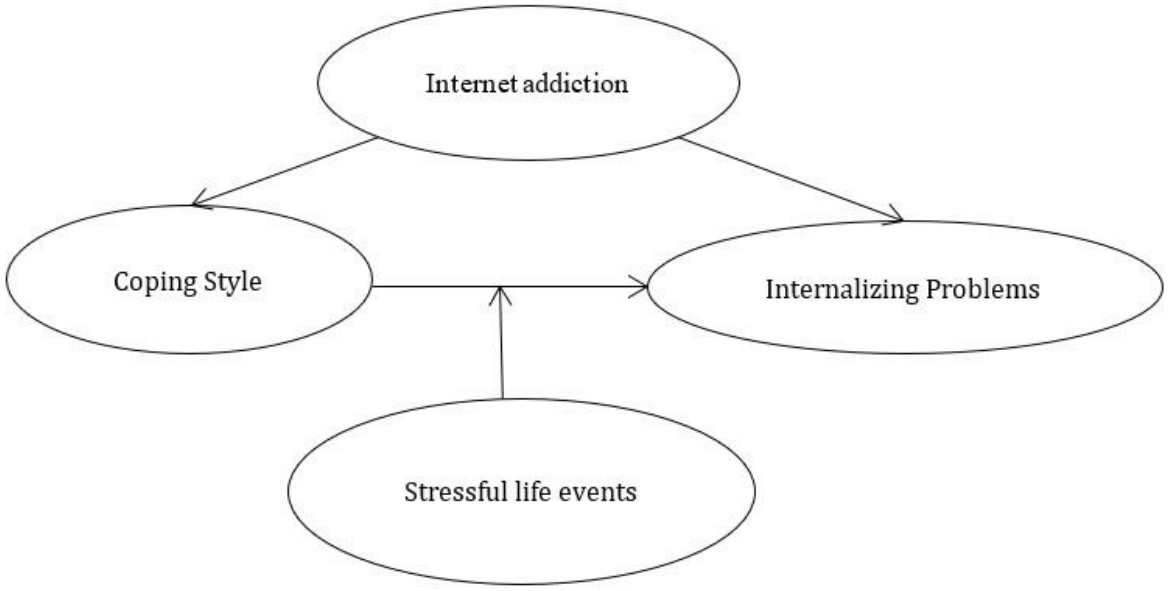

Figure 1. The hypothetical model for coping style, Internet addiction, stressful life events and internalizing problems.

METHODS

Subjects

Using convenient sampling method, 1639 students (average age 15.34 ± 1.81) were chosen from grade 7, 8, 10 and 11 of three junior high and high schools in Guangdong province, China. The questionnaire and anthropometric indices data were collected in the same week. The questionnaire has the self-evaluation format. After taking out the invalid questionnaires due to missing data or formatted answers, we collected 1601 valid questionnaires, with the effective collection rate of 97.68%. The same students were subjected

to anthropometric indices measurement, and we collected 1518 valid data points of the 1618 subjects tested, with an effective collection rate of 93.82%. After combining the questionnaire and anthropometric data, 1438 data points were eventually collected for data analysis. Among the subjects 627 were male while 811 were female; with 351 grade 7 students (24.41%), 341 grade 8 students (23.71%), 372 grade 10 students (25.87%) and 374 grade 11 students (26.01%). Among all subjects 57.16% were only child while the rest 42.84% have siblings. Basing on the education level of the subjects' parents, 4.2% fathers and 2.8% mothers do not have formal education or not graduated elementary school; 4.2% fathers and 7.8% mothers only graduated elementary school; 28.7% fathers and 34.8% mothers have senior high school level education; 47.9% fathers and 43.9% mothers have high school or equal level education; while 15% fathers and 11.4% of mothers have college or above level education.

Study Tools

Anthropometric indices and the selection of overweight/obese adolescents

Anthropometric indices include: Body Mass Index (BMI), Waist Circumference (WC), Waist-Hip Ratio (WHR), Waist-Height Ratio (WHTR), Systolic Blood Pressure, Diastolic Blood Pressure, Fasting Blood Glucose (FBG), Triglyceride (TG), Total Cholesterol (TC), High Density Lipoprotein Cholesterol, HDL-C, Low Density Lipoprotein Cholesterol (LDL-C) etc. All physiological and biochemical data collection was all performed by measurers that have undergone standard training. The height, weight, waist circumference and hip circumference were all measured in air-conditioned rooms at appropriate temperature. Students wore light underwear and were bare-foot for the measurements. Height and Weight were measured by automatic meters (Henggang SG, Shanghai, China). Systolic and diastolic blood pressure were measured by blood pressure monitor (Yutu, Shanghai, China). Students were fasted for at least 8 hours before blood collection. Blood biochemical data including blood lipids were measured by automatic biochemical analyzer (RiLi 7150, Tokyo, Japan). The criteria for overweight/obese adolescent selection were based on the Chinese children and adolescents BMI overweight, obesity screening classification criteria (WGOC).[34] The formula for BMI index is: weight (kg)/height (m^2).

Young Internet Addiction scale (Internet Addiction Test, IAT)

Young Internet addiction scale is also called the 'Internet Addiction Test', which is designed by Kimberly S. Young from University of Pittsburgh. The Chinese version of this form use DSM-IV as criteria, which is modified according to the diagnosis of pathological gambling addiction. This scale could be used for both adults and children, the questions were formatted like 'Do you think the time you spend online are longer than you expected?' There are 20 questions in total. All questions were scored 1-5 basing on the answers 'Never, Randomly, Sometimes, Usually, Always'. The higher sore indicates more severe case of Internet addiction. This test has accumulated steady reliability and efficiency, and it has shown high reliability and efficiency under different language environments.[19,21] This test has shown internal consistency coefficient of 0.91 in this study.

Trait Coping Style Questionnaire (TCSQ)

The trait coping style questionnaire used in this study is designed by Qianjin Jiang.[35] It is to reflect the coping strategies related to the relatively stable characteristics of individuals, and are associated with the individual's personality traits. This questionnaire includes 20 questions, which are scored 1-5 basing on the answers 'Never, Randomly, Sometimes, Usually, Always'. Two factors were extracted basing on the factor analysis, including negative coping styles (NC) and positive coping styles (PC). Individual that scored higher on one factor indicates that the individual has characters that are more prone to that corresponding coping style. NC and PC have internal consistence coefficient of 0.69 and 0.7. In this study NC and PC have internal consistence coefficient of 0.88 and 0.87, respectively.

Stressful life events scale

This stressful life events scale contains 82 subsets, which asks the possible life events experienced during the past half a year. These events are mainly categorized into family-related events, school-related events, social relationship-related events and personal events. All events were categorized as never experienced (score 0) or experienced, in the latter case the impact of this event will be asked and scored from 1-5 basing on the answers 'Severely affected, heavily affected, moderately affected, slightly affected, not affected'. The scores from each subset will be added up, the score higher than 1 indicated related events have been experienced. The higher the score obtained for an adolescent that has experienced stressful life events, the more moderate effect these events have on its mood. The sum-up value indicates the stress intensity level for each category. In this study, the internal consistence coefficient is between 0.92-0.94 for each category in this scale.

Self-rating depression scale, SDS

Self-rating depression scale is designed by Zung in 1965.[36] It is a scale used for counseling, depression symptoms screening and severity evaluation, as wells as for psychopharmacology research. The scale contains 20 subsets, 10 of them are designed for reverse scoring. It is scored 1-4 basing on the answers 'seldom or never, sometimes, often, most of the time or always'. Higher score indicates more severe depression symptoms. This scale has an internal consistence coefficient of 0.86. In this study, this coefficient value is 0.77.

Self-rating anxiety scale, SAS

Self-rating anxiety scale is designed by Zung in 1971.[37] It is quite similar to SDS, and is mainly used for evaluating the degree of severity. The scale contains 20 subsets, 5 of them are designed for reverse scoring. It is scored 1-4 basing on the answers 'seldom or never, sometimes, often, most of the time or always'. Higher score indicates more severe anxiety levels. This scale has an internal consistence coefficient of 0.78. In this study, this coefficient value is 0.74.

Data Analysis

Data were analyzed by SPSS 19.0 software. Since the number of missing data for major variables were limited, we estimated the missing values using the maximum likelihood estimation method. With the premise to protect the original data as much as possible, this approach would allow us to get better-unbiased parameter estimation and more accurate standard errors. First, all main variables were statistically described; Secondly, the relations between the main variables were analyzed through Pearson correlation; Finally, the moderated mediation model was analyzed by regression model. In addition, to efficiently reduce type I and type II errors if the premise of null hypothesis is not met in classic parameter tests (i.e. homogeneity of variance), we used the bootstrapping approach[38] for significant test of regression coefficient, in order to get the robust standard error and confidence intervals of parameter estimation. If the confidence interval does not include 0, it indicates there is statistical significant difference.

Table 1. Comparison of internalizing problems and anthropometric indices among adolescents with different body weight.

Variables	Normal weight group (n = 1193)	Overweight/Obesity group (n = 245)	*P*
Internalizing problem			
Depression (SDS)	2.16±0.41	1.84±0.42	0.001
Anxiety (SAS)	1.63±0.34	1.67±0.33	0.11
Obesity parameters			
BMI	18.48±2.04	25.12±2.76	0.001
Waist circumference (WC)	66.52±6.87	78.25±8.15	0.001
Waist-to-hip ratio (WHR)	0.81±0.07	0.86±0.06	0.001
Waist-to-height ratio (WHtR)	0.41±0.04	0.49±0.05	0.001
Biochemical parameters			
Triglycerides (TG)	0.96±0.397	1.44±0.741	0.001
Fasting blood-glucose (FBG)	4.61±0.41	4.65±0.45	0.53
Total cholesterol (TC)	4.05±0.693	4.46±0.846	0.001
HDL-cholesterol	1.38±0.274	1.28±0.222	0.002
LDL-cholesterol	2.48±0.614	2.89±0.843	0.001
Blood pressure			
SBP	106.86±8.536	112.04±7.327	0.001
DBP	65.92±6.515	69.49±6.608	0.001

RESULTS

Examining the Common Method Biases

Due to the fact that self-report was the only way of collecting data in this research, its results could be affected by common method biases. In order to eliminate these biases as much as possible, we strictly controlled the process of data collection and used Harman's single-factor test to limit the common method biases. Initially, during the data collection, we collected data anonymously, separated the questionnaires with similar contents, and subtly changed the description of questionnaires among different subjects. After data collection, we examined the common method biases with Harman's single-factor test. It turned out that rotated or not, the characteristic root of the eleven factors was always greater than 1. Meanwhile, the variance retrieved from the unrotated first factor was 21.01%, while it became 13.76% after rotation. Both of them were lower than the standard of 40%, which indicated that there are no severe common method biases in this research.

The Prevalence of Overweight and Obesity in Adolescents

According to WGOC BMI standard, all subjects were divided into two groups: 82.96% into the normal weight group (BMI 18.48±2.04 kg/m^2, n = 1193) and 17.04% into the overweight/obesity group (BMI 25.12 ± 2.76 kg/m^2, n = 245). The difference between these two groups was significant in BMI ($t = 35.26$, $P < 0.001$) as well as many other indicators of obesity waist circumference, waist-to-hip ratio, waist-to-height ratio and biochemical parameters (including TG, TC, HDL-C, LDL-C, Ps < 0.001) (See **Table 1**). We compared the normal weight group to the overweight/obesity group to determine whether the difference in internalizing problems (e.g. depression, anxiety) was significant. The results showed that these two groups have a significant difference in depression scores ($t = 2.95$, $P < 0.01$), while the difference in anxiety scores is not significant.

Descriptive and Correlational Analyses

The means, standard deviations and correlation matrix of variants are shown in **Table 2**, in which the mean values indicate the average scores of overweight/obese adolescents in different questionnaires. We found that there is a significant positive correlation between the internalizing problems in overweight/obese adolescents and three factors, which include Internet addiction, negative coping styles (NC), and stressful life events. This suggests that these three factors are risk factors for the internalizing problems in overweight/obese adolescents. Though positive coping style (PC) is significantly and negatively correlated with depression and anxiety, it doesn't have any significant correlation with Internet addiction and stressful life events. According to Wen, Hou, and Zhang,[39] a certain variant can't be a mediator when its correlation with the dependent/independent factor is not strong enough. Therefore, in our following mediation analysis, we only examined the mediation effect of negative coping style.

Table 2. Descriptive analysis and correlation matrix.

Variables	*M*	*SD*	1	2	3	4	5	6
1. Internet addiction	1.63	0.61	1.00					
2. stressful life events	9.63	2.70	0.36***	1.00				
3. positive coping style	3.11	0.97	0.12	0.09	1.00			
4. negative coping style	2.19	0.84	0.48***	0.35***	0.27***	1.00		
5. depression	1.96	0.41	0.23***	0.15*	-0.49***	0.18**	1.00	
6. anxiety	1.63	0.34	0.36***	0.21**	-0.19**	0.38***	0.60***	1.00

*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

The Relationship Between Internet Addiction and Internalizing Problems in Overweight/Obese Adolescents

We used hierarchical regression to examine the mediating model of Internet addiction and internalizing problems in overweight/obese adolescents, which is moderated by negative coping styles and stressful life events. According to Hayes;[40] Muller, Judd and Yzerbyt;[41] Wen and Ye,[42] in order to test for a moderated mediating model, we need to examine the first stage, second stage and direct effect of the moderation model to investigate whether they are influenced by the moderator variables. Determined by our hypothesis, this research only needs to examine the moderating effect of moderator variables in the second stage. Parameter estimates for three regression equations are necessary to test the moderated mediating model (See **Table 3**). Equation 1 estimates the effect of stressful life events (moderator variable) on the relationship between Internet addiction (independent variable) and depression/anxiety (dependent variable); equation 2 estimates the effect of stressful life events (moderator variable) on the relationship between Internet addiction (independent variable) and negative coping style (mediator); equation 3 estimates the effect of stressful life events (moderator variable) on the relationship between negative coping style (mediator) and depression/anxiety (dependent variable). If the regression test validates the effect of mediators as well as the moderating effect of moderator variable, the existence of a moderated mediation is proved. This study centers all predictors in every equation and controls factors including sex, age, and parent's education level to avoid multi-collinearity.

As shown in **Table 3**, in equation 1, the main effect of Internet addiction (IA) on internalizing problems in overweight/obese adolescents is significant ($\beta_{depression} = 0.22$, $\beta_{anxiety} = 0.24$, Ps < 0.01), whereas the main effect of stressful life events (SLEs, $\beta_{depression} = 0.02$, $\beta_{anxiety} = 0.61$, Ps > 0.05) and the interaction effect of these two ($\beta_{depression} = 0.07$, $\beta_{anxiety} = 0.003$, Ps > 0.05) are insignificant. In equation 2, both IA and SLEs significantly and positively predict negative coping style (NC, $\beta1 = 0.84$, $\beta2 = 0.08$, Ps < 0.01), and the interaction effect is significant as well ($\beta = 0.03$, Ps < 0.05). In equation 3, NC has a significant main effect on internalizing problems in overweight/obese adolescents ($\beta_{depression} = 0.19$, $\beta_{anxiety} = 0.05$, Ps < 0.05), and the moderator of NC and SLEs can also significantly predict internalizing problems ($\beta_{depression} = 0.02$, $\beta_{anxiety} = 0.01$, Ps < 0.001). Comparing equation 3 to equation 1, we found that $\Delta R^2_{depression} = 0.02$, $\Delta R^2_{anxiety} = 0.03$, which explain 2% and 3% of the deviation respectively.

Table 3. Testing of the moderated mediating model for the effect of Internet addiction on internalizing problems in overweight/obese adolescents.

	Depression						Anxiety					
	Equation 1 Depression		Equation 2 Negative coping style		Equation 3 Depression		Equation 1 Anxiety		Equation 2 Negative coping style		Equation 3 Anxiety	
	β	*t*	*β*	*t*	*β*	*t*	*β*	*t*	*β*	*t*	*β*	*t*
Sex	-0.02	-0.29	0.41	4.81**	-0.05	-0.94	0.01	0.19	0.41	4.81**	-0.03	-0.70
Age	-0.03	-0.22	0.11	4.67**	-0.52	-1.28	0.02	1.26	0.11	4.67**	0.004	0.32
Father's education level	-0.01	-0.43	-0.03	-0.75	-0.01	-2.06	0.002	0.11	-0.03	-0.75	0.01	0.28
Mother's education level	-0.04	-0.14	0.06	1.27	-0.05	-1.47	-0.06	-2.32	0.06	1.27	-0.07	-2.58
Internet addiction (IA)	0.22	1.94**	0.84	4.64***	018	1.37	0.24	2.75**	0.84	4.64***	0.18	1.78*
Stressful life events (SLEs)	0.02	1.19	0.08	2.83**	0.01	0.36	0.61	1.33	0.08	2.83**	0.07	0.49
IA×SLEs	-0.07	-0.70	-0.03	-1.99*	-0.01	-0.62	0.003	0.88	-0.03	-1.99*	-0.01	0.52
Negative coping style (NC)					0.19	3.65**					0.05	1.71*
NC×SLEs					0.02	3.76***					0.01	3.77***
R^2	0.06		0.39		0.08		0.14		0.39		0.17	
F	3.19**		23.48***		3.14***		6.68***		23.48***		6.41***	

Our data suggests that NC is a partial mediator between IA and internalizing problems, and SLEs have a moderating effect on the relation between NC and internalizing problems. Subsequently, we used the simple slope test[43] to further analyze the moderating effect. We analyzed the effect of NC on internalizing problems in overweight/obese adolescents at two conditions, i.e. SLEs = M + SD and SLEs = M - SD respectively (See **Figure 2** and **Figure 3**). Results show that

for those with a high level of stressful life events, NC has a significant positive predictive value on depression/anxiety in overweight/obese adolescents ($b_{depression}$ = 0.43, $t_{depression}$ = 7.34, $b_{anxiety}$ = 0.36, $t_{anxiety}$ = 5.57, Ps < 0.001); for those with a low level of SLEs, NC has a weaker (simple $slope_{depression}$ = 0.43 decreases to simple $slope_{depression}$ = 0.10; simple $slope_{anxiety}$ = 0.36 decreases to simple $slope_{anxiety}$ = 0.12) yet positive effect on depression/anxiety in overweight/obese adolescents ($b_{depression}$ = 0.10, $t_{depression}$ = 2.35, $b_{anxiety}$ = 0.12, $t_{anxiety}$ = 2.82, Ps < 0.05). This indicates that with the increase in stressful life events, the effect of NC on depression/anxiety in overweight/obese adolescent increases as well. In other words, the indirect impact of Internet addiction on internalizing problems in overweight/obese adolescents increases with the level of stressful life events (with a mediator of negative coping style).

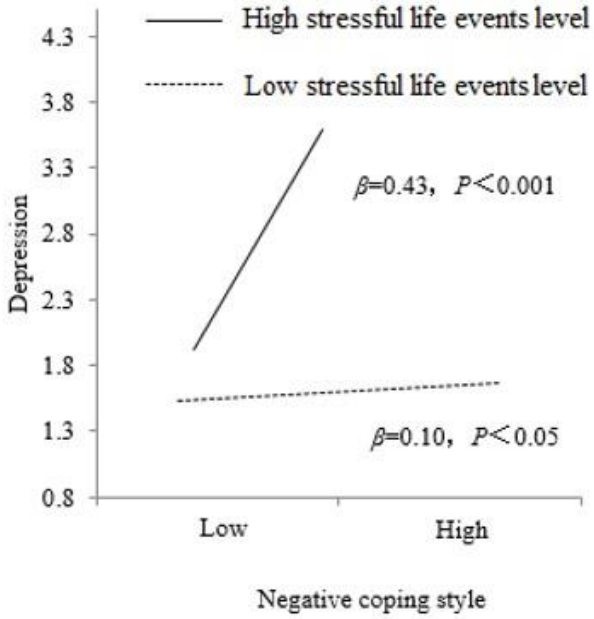

Figure 2. The moderating effect of stressful life events on negative coping style and depression.

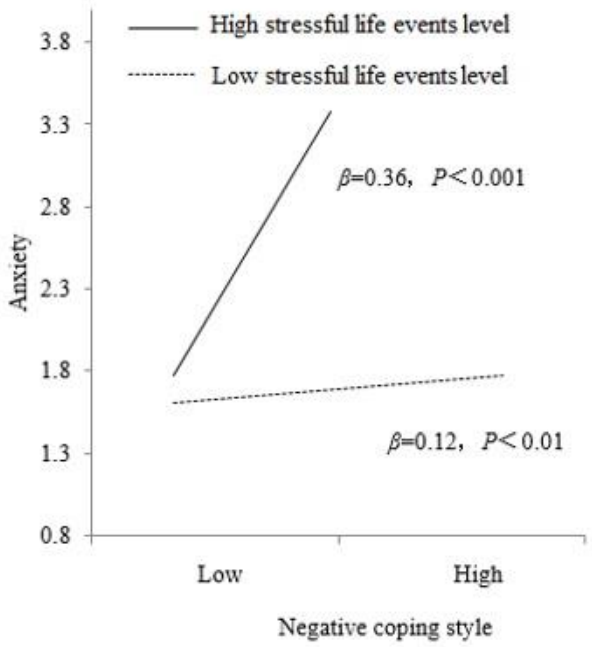

Figure 3. The moderating effect of stressful life events on negative coping style and anxiety.

DISCUSSION

The Mediating Effect of Negative Coping Style

Based on the existing research, this study includes coping styles to explore the mechanism of Internet addiction regarding its influence on the internalizing problems in overweight/obese adolescents. The result showed that the negative coping style partially mediated the impact of Internet addiction on internalizing problems in overweight/obese adolescents, which preliminarily verified the hypothesis of this research - the mediating effect of coping style. Davis[28] and social-cognitive theory suggest that the impact of Internet addiction on individual problem behavior is mainly through intrinsic characteristics of individuals, coping styles and other internal factors. The results of this study not only support this theory, but also prove that negative coping style is an important mediator in the relationship between Internet addiction and internalizing problems among overweight/obese adolescents.

This study found that Internet addiction was significantly positively correlated with internalizing problems in overweight/obese adolescents, indicating that Internet addiction is a risk factor for depression and anxiety. Individuals with a strong Internet addiction are more likely to suffer from depression or anxiety problems, which is in line with the previous studies.[11,14,21] At the same time, negative coping style also has a significant direct predictive value on depression and anxiety, which is consistent with Beck's Cognitive Susceptibility Model for Depression and Development. That is, stress can activate individual susceptibility (mainly due to attribution style, personality, self-cognition assessment and coping style, etc.), which leads to the internalizing problems such as depression.[44] As for overweight/obese adolescents, they are more prone to emotional problems such as depression and anxiety, because they hope to alleviate negative emotions in this way. Therefore, individuals who are more inclined to adopt passive coping strategies such as avoidance and withdrawal are more likely to form such a problem handling mechanism. This study further examines the indirect effect of Internet addiction on the internalizing problems in overweight/obese adolescents through passive coping style and finds that individuals with severe Internet dependence lack effective coping strategies

when facing stress problems such as frustration or stress. They are less likely to change the stress environment by solving problems and asking for help. Instead, most of them adopt negative coping styles such as withdraw, self-blame and fantasy, causing negative behavioral and emotional problems such as hyperphagia, loneliness, depression, and anxiety.[45,46] Therefore, when facing the society in real life, they feel more and more isolated and tend to escape the reality through Internet, which eventually becomes a vicious cycle.

The Moderator Effect of Stressful Life Events

In this study, regression analysis found that stressful life events can regulate the indirect effects of Internet addiction on internalizing problems in overweight/obesity adolescent. The specific regulatory role is located in the second half of the intermediary chain. That is, the relationship between the negative coping style and the internalizing problems in overweight/obese adolescents is conditioned by the stressful life events they experience. The indirect effects of Internet addiction on the internalizing problem in overweight/obese adolescents by negative coping patterns increase with the growing level of stress life events. This result validates our assumptions. In the meantime, Wagner's[33] stress-coping model is further verified and expanded. That is, addiction behavior is a coping reaction of addicts to cope with stressful events. At the same time, in the process of Internet addiction, in view of the dual effects of pulling force (temptation felt by the Internet users) and pushing force (life event stress), overweight/obese adolescents need two sets of independent psychological mechanisms to cope with the synergistic effect of the two: the need of "stress coping system" to deal with life events stress in order to solve or reduce the negative influence; the need of “temptation coping system” in face of the perceived Internet temptation to prevent the Internet addiction. These two coping systems are independent of each other, even though individuals resist the temptation from the Internet to ease the stress of life events, they may still indulge in the network to escape the negative emotions and adverse effects in real life because of the immature coping styles (such as fantasy, withdraw, self-blame can also include the rationalization etc.) they use.[26] Therefore, the coping style cannot independently restrain Internet addiction and internalizing problem. It needs to work together with stressful life events. When the amount of stressful life events accumulates, overweight/obese teenagers are more inclined to adopt a negative coping style, and more likely to rely on the Internet to relieve their own stress, resulting in depression, anxiety, and other internalized behavior. Thus, stressful life event is a positive moderator in the indirect effect of Internet addiction on internalizing problems in overweight/obese adolescents.

Research Value and Prospects

This study reveals the intrinsic mechanism of Internet addiction on the internalizing problems in overweight/obese adolescents, which is of both theoretical and practical value. First of all, in theory, this study not only helps to understand how Internet addiction acts directly and indirectly on the internalizing problems in overweight/obese adolescents, but also further reveals the strength differences on individuals who experience different levels of life events and being influenced by mediation effects.[47] Secondly, this study provides empirical evidence on the mechanism of internalizing problem formation of overweight/obese adolescents. In fact, the discussion on the mechanism of internalizing problems in overweight/obese adolescents has important implications for the prevention and intervention of adolescent overweight/obesity, depression and anxiety. Although individuals with high levels of Internet dependence are more likely to develop depression, anxiety, and other negative emotions, the possibility of internalization can be reduced through intervention in predisposition such as self-awareness assessment and coping style. By increasing the awareness of overweight/obese adolescents, admitting themselves and making themselves believe that they can handle the stressful life events, they should take more active measures to actively seek support and help. In addition, the living and learning environments of adolescents should also be optimized to minimize the frequency of negative life events. More importantly, schools should make use of various channels to conduct extensive mental health education and psychological counseling among adolescents to improve their ability to cope with negative life events, regulate the role of Internet addiction on the issue of internalization and reduce the possibility of internalizing problem formation of overweight/obese adolescents.

This study also has some limitations. First of all, the self-report method was used to collect the data. Although the results of Harman's single-factor test showed that there were no significant common method biases, there may still be some social appraisal effect. Due to smaller sample sized survey, the interpretation is limited because there are much bias and confounding factors in this cross-sectional study. It is necessary for the future research to collect the data through various means, such as teacher's report, parent's report and peer nomination, in order to further improve the validity of the research. Secondly, due to the cross-sectional study design, the relationship between variables could not be clarified by causal inference. In the future, it is necessary to use the method of longitudinal follow-up research to further verify the research results. Finally, in this study, overweight and obesity subjects were screened only with anthropometric parameters, and the clinical symptoms of obesity were not diagnosed. Therefore, in the future, it is necessary to study the clinically diagnosed obesity adolescents so as to make the research results more clinically significant.

CONCLUSION

(1) Internet addiction has a direct impact on the internalizing problems in overweight/ obese adolescents, and indirectly affects them through coping styles. The negative coping style plays a partial mediating role in the problem of internalization of overweight/obese adolescents by Internet addiction.

(2) The mediating effect of negative coping styles was moderated by stressful life events.

CONFLICT OF INTEREST
None.

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Review

ASD Pathogenesis and Emerging Treatments: Lessons Learned from the Monogenic Syndromic ASD

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Autism Spectrum Disorder (ASD) is a group of complex neurodevelopmental disorders characterized by social impairments and repetitive behaviors. It can be divided into two major subcategories: 1) non-syndromic (sporadic or idiopathic) ASD and 2) syndromic ASD that also manifests other characteristic medical conditions and physical features. ASD is a growing public health crisis as its prevalence increases rapidly in recent years. There is no FDA approved drug for the treatment of ASD core symptoms that define the disorder, which is a major challenge in the management of ASD. This is largely due to the lack of a good understanding of its etiology that is highly complex and heterogeneous. Many types of the syndromic ASD are caused by mutations of a single gene (monogenic), which provides an excellent tool to explore the disease mechanisms leading to the pathogenesis of the core symptoms. Here, we briefly review the recent progress in animal studies on the disease mechanisms of the fragile X syndrome and the syndromes caused by loss of function of a key negative regulator along the mTOR signaling cascade due to the deleterious mutations of the respective gene. We emphasize the disrupted signaling pathways likely shared by some non-syndromic ASD cases, and highlight druggable targets and their translation for the treatment of ASD patients.
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Key Words: *ASD, neurodevelopmental disorders, monogenic syndromic ASD*

ASD: A GROWING PUBLIC HEALTH CRISIS

ASD is a range of complex neurodevelopmental disorders characterized by impairment of social interactions and restricted interests, stereotyped and repetitive behaviors.[1-4] In addition to the core symptoms, there are other common co-existing psychiatric and medical conditions in individuals with ASD, such as epilepsy, intellectual disability (ID), anxiety, aggression, sleep and gastrointestinal problems.[5] The prevalence of ASD in the world is estimated to be more than 1%.[6] The prevalence increases rapidly in recent years. According to the 2016 report prepared by the US Center for Disease Control and Prevention (CDC) (based on the nationwide data collected in 2012),[7] the prevalence has increased by 130% from 2002 to 2012. Currently, ASD affects 1 in 68 children aged 8 years in the US.[7] Up to two thirds of ASD individuals have low daily living skills, and need life-time care by their family members or other caregivers.[8] Thus, ASD is a significantly more serious challenge to the society than the Alzheimer's disease, as the latter usually affects only the last decade of the patient's life.[9]

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WIDENING KNOWLEDGE GAP BETWEEN ASD GENETICS AND HOW THE AFFECTED PROTEINS CONTRIBUTE TO ASD PATHOGENESIS

There is no approved drug for the treatment of the core symptoms of ASD, which is a major challenge for its management. This is largely because ASD is a group of highly heterogeneous disorders and our current understanding of their etiologies is still very limited. Rapid advances in sequencing technologies and drastic drop of the cost have made it possible to sequence large samples of normal individuals and those with ASD, which allows to identify mutations of ASD-linked genes even with very low penetrance (< 1%).[10,11] As of July 10, 2017, 910 ASD risk genes have been suggested based on human and animal studies (https://gene.sfari.org/). However, while the discovery of ASD risk genes is at an increasingly rapid pace, our understanding of how mutations of these risk genes contribute to ASD pathogenesis lags further behind.

A gene mutation can lead to at least four possible functional outcomes of the affected protein: 1) reduced or loss of function (especially when the protein fails to express); 2) increased function; 3) no change in function; and 4) gain of different (new) function. A deeper understanding of mutation-induced functional alterations of the gene products (usually proteins) and how these alterations contribute to the disease mechanisms, are prerequisites to develop novel disease-modifying pharmacotherapies for the treatment of ASD. The sequencing technology revolution makes gene mutation

detection significantly easier than ever before, but understanding how a particular mutation changes the function of the affected protein and how this functional change contributes to ASD pathogenesis could take many years and requires collaborative efforts of many research labs in the world.

Searching for additional ASD risk genes and mutations is important in the long run to fully understand the etiology of ASD and the related disorders. However, it seems to be equally important to allocate enough resources in order to digest and integrate the overwhelming and highly informative data gathered already from the ASD genetics[10-12] and numerous animal models,[12] to delineate how the identified deleterious mutations of these 910 risk genes alter the function of the affected proteins and cause ASD.

MONOGENIC SYNDROMIC ASD AS A POWERFUL TOOL FOR EXPLORATION OF ASD PATHOGENESIS

ASD can be divided into two major subcategories: syndromic and non-syndromic (also called idiopathic or sporadic) ASD.[12-13] The etiology of most non-syndromic ASD cases is complex, believed to be due to interactions between low penetrant mutations of multiple risk genes and environmental factors.[11,14] It is practically hard to generate animal models to study the disease mechanisms in this situation.

In the syndromic ASD, in addition to the core symptoms, there are other characteristic neural and non-neural conditions (particularly dysmorphic features) not seen in the non-syndromic ASD. Many types of syndromic ASD are caused by highly penetrant mutations of a single ASD gene (monogenic, see below). This provides an excellent opportunity to explore the disease mechanisms of ASD and to identify druggable targets for the development of novel treatments. In the sections below, we review several well-studied monogenic syndromes associated with ASD and describe some common disease mechanisms that are likely shared by some non-syndromic ASD cases. We also discuss the mechanism-based investigational treatments tested in animal models and evaluated in some preliminary clinical studies. Due to space limit, we focus only on those syndromic ASD subtypes in which excessive protein synthesis is the main cause of the syndrome due to the loss of a key negative regulator.

FRAGILE-X SYNDROME

Fragile X Syndrome (FXS), an X-linked disorder, is caused by mutations in the *FMR1* gene, which results in a significantly reduced or even completely loss of the expression of Fragile X mental retardation protein (FMRP).[15] It is the most common inherited form of ASD, found in ~2-6% of individuals with ASD.[16-17] FXS is probably also the most extensively studied syndromic ASD so far. The knowledge gained from these studies is highly valuable to elucidate ASD pathogenesis in general. For this reason, we use more space to describe the disease mechanisms of FXS than those for three other syndromic ASD subtypes covered in this review.

In the full mutation group (FMRP is not expressed), the ASD phonotypes are found in nearly 70% male patients. In premutation males (FMRP expression is reduced), the ASD incidence drops quite significantly to 14%,[18] indicating a causal relation between the FMRP deficiency and ASD pathogenesis. Other common medical manifestations include seizure, aggression, attention deficit hyperactivity disorder (ADHD) symptoms, anxiety, sensory hypersensitivities, self-injury, macrocephaly, and sleep disturbance. These are also comorbid conditions frequently observed in non-FXS ASD patients.[5,19] Knocking out (KO) *Fmr1* gene in animal models produces phenotypes that manifest virtually all the major clinical and neuroanatomical features, such as cognitive impairment, autistic behaviors, macroorchidism, macrocephaly, immature spines on the dendrites [16-20] found in human patients, which further confirms the causal relation between the loss of FMRP and FXS symptoms.

FMRP AND TRANSLATION OF THE PROTEINS INVOLVED IN SYNAPSE FORMATION, MATURATION, AND FUNCTION

FMRP is a master negative regulator (repressor) of the cap-dependent protein translation. It binds to its target mRNAs and controls the initiation of the translation process.[21-22] FMRP is highly expressed in neurons. It regulates local protein synthesis in a rapid and activity-dependent manner, particularly in dendritic spines where synapses are located.[23-24]

Many FMRP target mRNAs encode proteins (such as neuroligins, neurexins, shanks, MMP-9, and mGluR5) required in synapse formation, maturation, elimination, and neural plasticity.[25] Disruptions of these synaptic processes are linked to ASD.[14] Furthermore, many protein partners involved in FMRP-required translation control and signaling pathways are themselves encoded by known ASD-linked genes, such as *eIF4E*, *MEF2C*, *PCDH10* and *CYFIP1*,[26-28] and deleterious mutations of these genes have been found in non-syndromic ASD patients.[26-28] This suggests that dysfunction of the FMRP-dependent translational control and signaling has a broad significance in understanding not only the pathogenesis of FXS, but also that of some non-syndromic ASD cases. We use two representative FMRP regulated proteins (**Figure 1**) to demonstrate that an excessive translation of the proteins involved either in synapse maturation or elimination is linked to ASD pathogenesis.

EF1α OVER EXPRESSION DUE TO FMRP DEFICIENCY DISRUPTS SYNAPSE ELIMINATION

Sensory experience-dependent synapse consolidation and elimination during postnatal neurodevelopment are critical processes to shape and refine specific and mature brain circuits.[29] Through controlling translation of a regulatory protein, EF1α (eukaryotic translation elongation factor 1-α), FMRP plays a key role in activity-dependent, MEF2 (myocyte enhancer factor 2)-initiated elimination of excitatory synapses.[27]

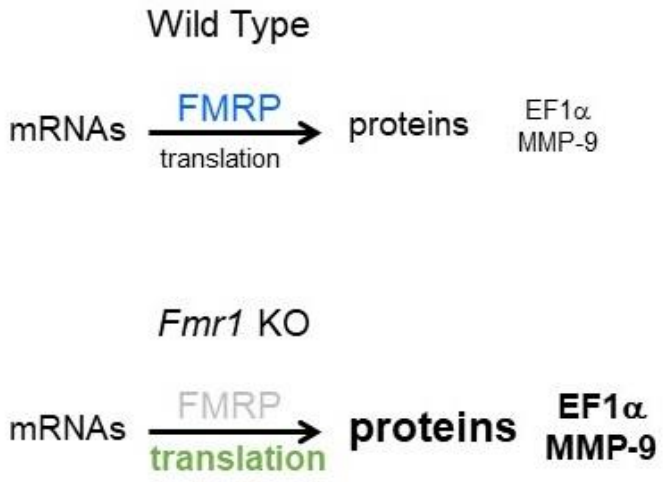

Figure 1. Loss of FMRP-dependent control in protein translation leads to an excessive translation of some proteins, such as MMP-9 and EF1a, that are critically involved in synaptic maturation and elimination.

MEF2 is a transcription factor and is activated by neural activity.[30] It triggers excitatory synapse elimination by promoting Pcdh10-dependent degradation of PSD-95 (a main postsynaptic scaffolding protein in excitatory synapses), which is a key step in the activity-dependent elimination of the excitatory synapse (**Figure 2A**). In wild-type (WT) neurons, MEF2 activation induces 1) translocation of Mdm2 (murine double minute 2, a specific ubiquitin E3 ligase for PSD-95) from the dendrite to the spine, and 2) transcription of *Pcdh10* gene. Pcdh10 facilitates the proteasomal deposition of PSD-95 after its ubiquitination (Ub-PSD-95 in Figure 2A) by Mdm2, leading to degradation of PSD-95 and eventual elimination of the synapse.

In *Fmr1* knockout neurons (**Figure 2B**), the loss of translational control due to FMRP deficiency causes over production of EF1α, which binds specifically to Mdm2 and prevents it from translocating to the spine. This prevents PSD-95 ubiquitination and degradation, which stabilizes the excitatory synapse.

The discovery of this synapse elimination pathway *enriched* with multiple known ASD-linked genes (*FMR1*, *MEF2C* and *PCDH10*) has an important implication in the understanding of ASD pathogenesis. Deletion mutations of *MEF2C* and *PCDH10* genes have been identified in some rare sporadic ASD cases in human patients,[31-32] but how these mutations cause ASD is unknown. From the roles of MEF2 and Pcdh10 identified in this pathway, ASD may be caused by a disruption of excitatory synapse elimination during the early neurodevelopment in these patients. It is particularly interesting that a similar disruption in excitatory synapse elimination during early neurodevelopment due to heterozygous deletion of *Tsc2* gene in the animal model of tuberous sclerosis complex (TSC, see the corresponding section below) is also implicated in ASD pathogenesis.[33] Thus, disruption of synapse elimination is likely an important shared mechanism in the pathogenesis of both syndromic (FXS, TSC) [27,33] and some non-syndromic ASD cases.[31-32]

MMP-9 PROTEIN OVER EXPRESSION DUE TO FMRP DEFICIENCY LEADS TO ASD PHENOTYPES

MMP-9 (matrix metalloproteinase 9) is an extracellular proteinase that is translated in the dendritic spines and released from the excitatory synapses in response to neuronal activity. It is involved in the regulation of synaptic plasticity, learning and memory.[34] MMP-9 is required for growth and maturation of the dendritic spines and is implicated in pathogenesis of human epilepsy, FXS, and psychiatric conditions.[35] Translation of MMP-9 mRNA is repressed by FMRP and stimulated by eIF4E (eukaryotic translation initiation factor 4E) activation.[36]

Indeed, FXS (*Fmr1* KO) mice display increased MMP-9 activity [37] and this is due to loss of repressive translational control by FMRP.[38] Pharmacologically down-regulating the expression [36-37] or genetic removal [39] of MMP-9 in FXS models rescues the FXS symptoms and anatomical abnormalities, including social impairment, repetitive behavior, delayed dendritic spine maturation and even macroorchidism in the animal models of FXS. MMP-9 overexpression in wild type mice produces several FXS-like phenotypes.[36] This indicates that over expressed MMP-9 due to loss of translational control by FMRP plays a key role in FXS pathogenesis. Indeed, minocycline, an antibiotics that lowers MMP-9 level in FXS mice [40] also reverses synaptic and behavioral abnormalities in the FXS mice.[37] More importantly, it also demonstrates some clinical benefits in FXS patients.[41]

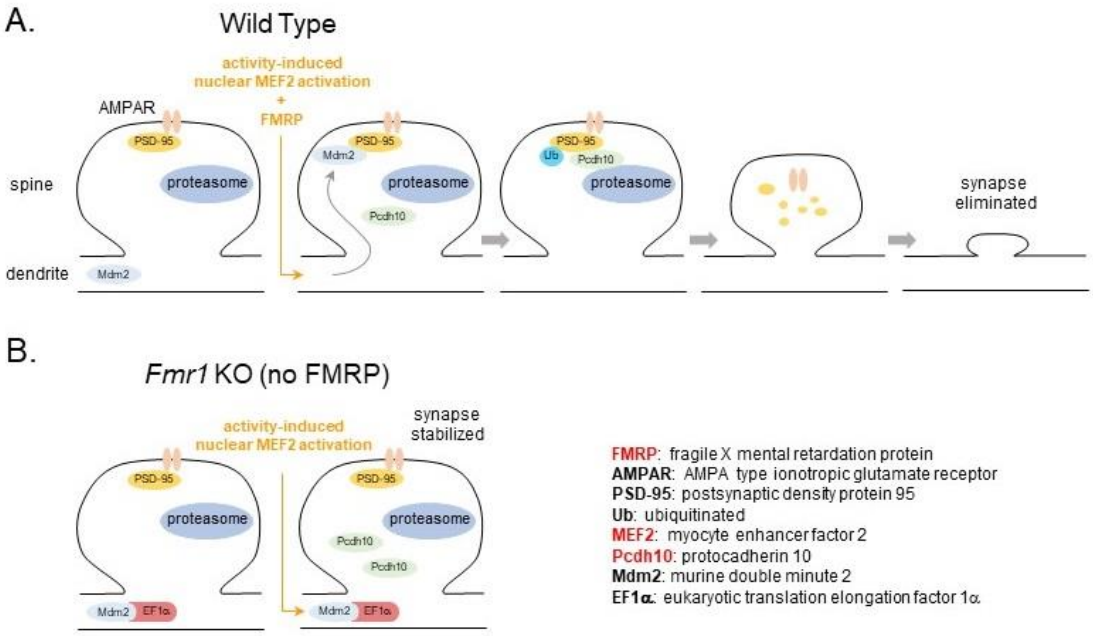

Figure 2. MEF induced, FMRP required activity-dependent elimination of excitatory synapse. **A**. In the wild type neuron, neural activity activates MEF2 that in turns stimulates Pcdh10 transcription and translation cooperatively with FMRP. MEF2 also causes translocation of Mam2 to the spine where it ubiquitinates PSD-95, allowing Pcdh10 to facilitate proteasome-mediated PSD-95 degradation and synapse elimination. **B**. The loss of FMRP-mediated translational control causes over expression of EF1a, which sequesters Mdm2 and prevents Mdm2-mediated PSD-95 ubiquitination and synapse elimination. This diagram is modified from ref. 27.

SYNDROMIC ASDS ASSOCIATED WITH OVERACTIVE MTOR, EXCESSIVE GROWTH, AND PROLIFERATION

mTOR (mammalian target of rapamycin) is a protein kinase. It usually forms two complexes, mTORC1 and mTORC2 ('C' stands for 'complex'), with two different groups of protein partners.[42] Here we only focus on the mTORC1 since its overactivation is closely associated with three types of syndromic ASD described in the next three sections.

The PI3K (phosphatidylinositol 3-kinase) -mTOR and RAS-ERK (rat sarcoma protein-extracellular signal-regulated kinase) signaling cascades are the two major pathways for controlling neuronal survival, growth, differentiation, proliferation, activity-dependent synaptic maturation and remodeling in response to extracellular signals such as neurotransmitters and neurotropic factors (**Figure 3**).[42-43] These two pathways converge to stimulate protein translation by phosphorylation of eIF4E (eukaryotic translation initiation factor 4E), 4EBP (eIF4E binding protein), and S6K (ribosomal S6 kinase). Activation of eIF4E (phosphorylated by MNK, **Figure 3**) directly stimulates the initiation of protein translation.[36,44] 4EBP is a specific inhibitory protein of eIF4E. When it is dephosphorylated it binds to eIF4E to suppress eIF4E function. When it is phosphorylated by mTOR, 4EBP dissociates from eIF4E, allowing for the initiation of translation by eIF4E.[44] Phosphorylation of S6K by mTOR induces protein synthesis at the ribosome, which could be a different type of protein translation from the one repressed by FMRP.[45] There is some evidence that activated (phosphorylated) S6K can also phosphorylate FMRP to enhance its function as a translational repressor [46] (**Figure 3**). The importance of this "push-pull" effect of S6K on protein synthesis is unclear, although it could render a more precise control of different translational machineries allowing specific sets of mRNAs be translated in response to selective activation of PI3K-mTOR versus RAS-ERK pathways.

Along these pathways, a number of inhibitory proteins control the activity level at several critical check points to ensure a balanced protein translation appropriate to functional need (**Figure 3**). These proteins are encoded by highly penetrant autism-linked genes. Loss of function mutations in *TSC1*, *TSC2*, *PTEN*, and *NF1* genes (encoding TSC1, TSC2, PTEN, and NF1 proteins, respectively), or gain of function mutations of *EIF4E* gene (encoding eIF4E) result in excessive protein translation and growth that disrupts synapse function, leading to syndromic and non-syndromic autisms (discussed in the next few sections below).

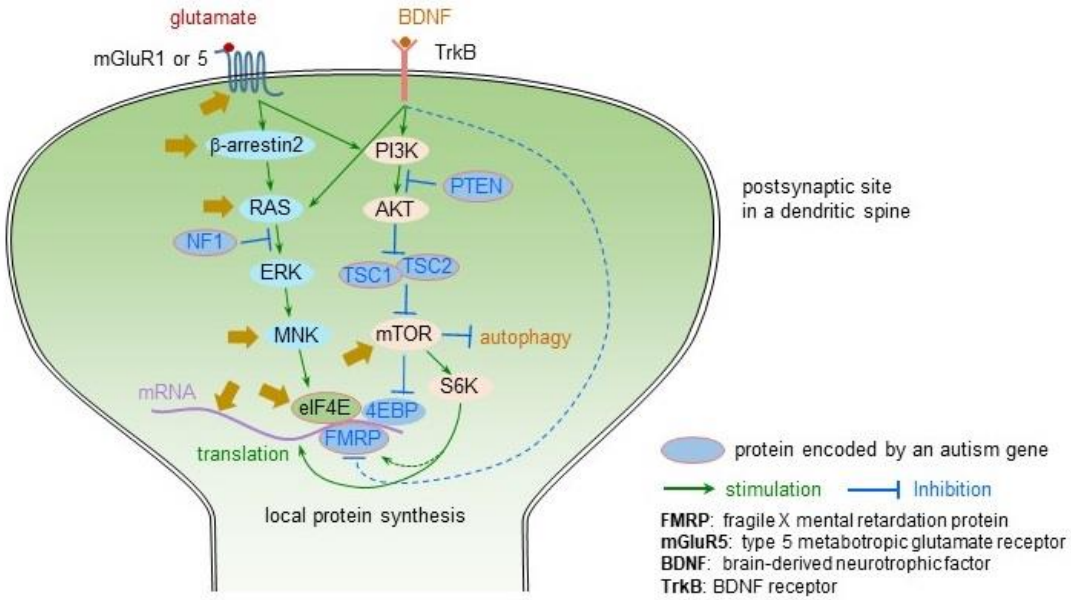

Figure 3. Neural activity activates the group1 mGluRs and TrkB receptors to stimulates local (synaptic) protein synthesis, which is stimulated mainly by the RAS-ERK and PI3K-mTOR pathways. The large arrows indicate potential drug targets.

TUBEROUS SCLEROSIS COMPLEX

Tuberous sclerosis complex (TSC) is a syndromic ASD. It is caused by autosomal dominant, loss of function mutations in *TSC1* or *TSC2* tumor suppressor genes, which results in an enhanced activation of the PI3K-mTOR pathway and dysregulation of cell growth and proliferation.[47] The abnormally elevated mTOR activity has a profound effect in neurodevelopment.[42,48] Many of TSC children develop macrocephaly, seizure, intellectual disability, and up to 60% of the patients have the ASD phenotype.[47,49] In animal models of TSC, which carry heterozygous genetic deletions of *Tsc1* or *Tsc2*, macrocephaly, epilepsy, and other psychiatric abnormalities, including autistic behaviors have been observed.[50-52] Furthermore, at the cellular level, disruption of synapse formation, maturation, and activity-dependent neural circuit remodeling, particularly reduction in pruning (elimination) of dendritic spines, are also observed in the *Tsc1/2* deficient mice.[33] Dendritic spine pruning is believed to be regulated by autophagy, a process negatively regulated by mTOR.[53] Activation of mTOR pathway leads to a suppression of autophagy (Figure 3), leading to a decreased pruning of dendritic spines and a higher synapse density.[33]

The molecular genetics and studies on the Tsc1/2 deficient mice have identified mTORC1 as a drug target for the treatment of TSC. Indeed, selective mTORC1 inhibitors and autophagy inducer,[53] such as rapamycin,[43] ameliorates many functional abnormalities as well as normalizes dendritic spine pruning in TSC animal models.[33,54,55] The efficacy of rapamycin in animal models validates the target and indicates a central role of mTOR overactivation in TSC pathogenesis. Interestingly, rapamycin also has beneficial effects in a mouse model of non-syndromic ASD,[56] suggesting that excessive mTOR activity may also play a key role in non-syndromic ASD.

The animal studies reviewed above indicate that mTOR can be a disease-modifying drug target for TSC. Indeed, recent clinical studies in human TSC patients show that administration of everolimus, another mTOR inhibitor, significantly reduces seizure, anxiety, depression, and autistic behaviors in children and adolescents.[57,58] However, as mTOR inhibitors are immune suppressants, their use can increase the risk of infections.

MACROCEPHALY/ASD SYNDROME CAUSED BY PTEN DEFICIENCY

Macrocephaly/ASD syndrome is caused by heterozygous loss function mutations in the *PTEN* gene. It is an autosomal dominant disorder characterized by macrocephaly, abnormal facial features, and delayed and altered neurodevelopment resulting in autistic behavior and/or mental retardation.[59,60]

Selective inactivation of *Pten* in the cortex and hippocampus of juvenile mice recapitulate some major Clinical abnormalities observed in the macrocephaly/ASD syndrome patients, including social interaction and cognitive impairment, anxiety, and macrocephaly.[61] In the mutant mice, activity of the PI3K-mTOR pathway is significantly increased due to loss of negative control by PTEN. Furthermore, the complexity of

dendritic arborization and spine density of the affected neurons have increased,[61] similar to that observed in the TSC mice.[33] This could also be related to autophagy deficit due to chronically elevated mTOR activity.[33,53,62] PTEN deficient mice also show deficits in LTP (long-term potentiation) and LTD (long-term depression), two major forms of synaptic plasticity, and impaired spatial memory.[63] The anatomical, cellular, behavioral abnormalities and seizure in PTEN deficient mice can be ameliorated by specific mTOR inhibitors indicating that the mTOR pathway downstream of PTEN plays a critical role in causing these abnormalities,[62,64] as in TSC.

NEUROFIBROMATOSIS TYPE 1

Neurofibromatosis type 1 (NF1) is a common autosomal dominant genetic disorder. It is caused by mutations of the *NF1* gene that encodes neurofibromin (NF1 protein), a GTPase-activating protein negatively regulating RAS activity.[65] Up to 80% of children with NF1 have cognitive and behavioral problems [66] and about 25% of the patients have ASD core phenotypes.[67]

In *Nf1*$^{+/-}$knockout mice, loss of neurofibromin is associated with increased RAS-ERK pathway activity, decreased synaptic LTP and a pattern of cognitive impairment similar to the human phenotype.[68] The cognitive deficits in the NF1 animal models can be reversed by pharmacologically inhibiting RAS-ERK activity with statin drugs (e.g., lovastatin, simvastatin).[69] Translation of the results from the animal studies to NF1 patients have a mixed results.[70,71]

LESSONS LEARNED: MECHANISMS, DRUG TARGETS, AND POTENTIAL MECHANISM-BASED TREATMENTS

Studies on the animal models of the four syndromic ASDs reviewed here have uncovered three key druggable disease mechanisms: 1) excessive translation of the FMRP-repressed mRNAs, 2), constitutively activated PI3K-mTOR pathway, and 3) constitutively activated RAS-ERK pathway. Several drug targets have been identified based on these mechanisms and are indicated by the large arrows in **Figure 3**. These targets include mGluR 1/5, β-arrestin2, RAS, MNK, mTOR, eIF4E, and the protein translation process. These drug targets have all been validated either pharmacologically [36,44,54,55,72] with the respective inhibitors/antagonists or genetically [73] (in the case of β-arrestin2) in animal models of these four syndromic ASDs. For some of these targets, preliminary clinical studies on the investigational treatments through repurposing approved drugs have demonstrated beneficial effects on autistic and comorbid symptoms in the syndromic ASD patients.[57,77]

The preclinical studies on the disease mechanisms of the syndromic ASDs could also help to explain how highly penetrant mutations of some ASD-linked genes cause non-syndromic ASD. For example, disruption in synapse elimination during postnatal neurodevelopment plays a key role in ASD pathogenesis.[33] In a key pathway required in synapse elimination, FMRP works cooperatively with MEF2 and PCDH10.[27] This may explain the pathogenesis of those sporadic ASD cases associated with loss of function mutations in *MEF2C* and *PCDH10* genes.[31-32] Sporadic ASD cases associated with gain of function mutations in the *EIF4E* gene [74] provide another example. In this case, the mutations reduce the effectiveness of negative regulation of eIF4E by 4EBP and FMRP (**Figure 3**), leading to an excessive eIF4E initiated protein translation. Since eIF4E is at a convergence point of the three disease mechanisms (**Figure 3**), the mechanism-based treatments for FXS and mTOR related syndromic ASDs could also be applied to the sporadic ASD cases associated with the gain of function mutations in the *EIF4E* gene.

CONCLUSIONS AND FUTURE DIRECTIONS

Syndromic ASD makes up approximately 10% of all ASD cases.[75] From the above discussion of four subtypes of monogenic syndromic ASD (together they make up more than 60% of all syndromic ASD cases),[75-76] a coherent theme seems emerge: functional deficiency of key *negative* controllers (FMRP, TSC1, TSC2, PTEN, and NF1) in either translation regulation, or two fundamental signaling pathways (PI3K-mTOR and RAS-ERK) result in excessive protein synthesis, growth, dysregulated differentiation, maturation, neural remodeling and plasticity, and autistic phenotypes. The disease mechanisms could also help to explain the pathogenesis of some non-syndromic ASD cases.

While the mechanism-based investigational treatments in some preliminary clinical studies are promising, more large-scale, double blind, and placebo controlled clinical trials with a longer treatment duration are needed to confirm their efficacy in the relevant syndromic and non-syndromic ASD patients. Furthermore, a better selection of drug targets (for example, mGluR5 vs one of its downstream effectors)[73] for drug development may increase the therapeutic window (difference between therapeutic and toxic doses) and therefore the chance of success. Finally, as the causes of syndromic ASD are highly heterogeneous, pre-Clinical studies on the disease mechanisms of other monogenic syndromic ASDs, including many rare ones, should provide a more complete picture about the etiology of ASD in general.

CONFLICT OF INTEREST
None.

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Review

Current Treatments of Prader-Willi Syndrome: A Systematic Review

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Prader-Willi Syndrome (PWS) is a genetic imprinting disorder mainly caused by the absence of paternally expressed imprinted genes at 15q11.2-q13, maternal uniparental disomy (UPD) and imprinting defect. Typical features include hypotonia in early infancy, subsequent hyperphagia and morbid obesity, developmental delay and intellectual disability. The aims of this systematic review are to summarize the current knowledge of the treatments for PWS based on the clinical studies published from 2000 to 2017. We searched three main databases - PubMed, MEDLINE, and Scopus, and selected 34 out of 1139 articles initially identified for this review. We focused our discussions on the widely-accepted growth hormone (GH) treatment, and emerging investigational treatments oxytocin (OXT), anti-diabetes and anti-obesity drugs. In addition, early detection, early treatment, and combination therapies are proposed to assure a better outcome.

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Key Words: *Prader-Willi Syndrome (PWS), medication therapy, growth hormone (GH), oxytocin (OXT), diabetes medications, treatments for Prader-Willi Syndrome*

INTRODUCTION

As the first recognized human genetic imprinting disorder, Prader-Willi Syndrome (PWS) has an estimated prevalence in several studied populations of 1/10,000-1/30,000.[1] This multisystem genetic disorder could be caused by the absence of paternally expressed imprinted genes at 15q11.2-q13 through paternal deletion, maternal uniparental disomy (UPD) of chromosome 15, or an imprinting defect.[2] The characteristic features of PWS include severe hypotonia in early infancy, subsequent hyperphagia and morbid obesity from early-childhood, developmental delay, mild intellectual disability, and a distinct behavioral phenotype.[1,3]

For adult patients with PWS, the leading cause of death is complications of obesity.[4] Though its etiology has remained unclear so far, the cause of hyperphagia for patients with PWS is usually considered to be hypothalamic dysfunction, which is also responsible for growth hormone (GH), sex hormone, and thyroid-stimulating hormone (TSH) deficiencies.[3,5] Current treatments for PWS include: well established clinical use of Growth Hormone (GH), and emerging new therapies such as Oxytocin (OXT), and drugs for diabetes mellitus and obesity such as Metformin, Beloranib, and GLP-1 receptor agonist. The objective of this systematic review is to summarize the most effective treatments for PWS based on clinical trials.

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METHOD

Research Design

A systematic review protocol was developed to explore the most promising treatments for PWS. The evaluation of different categories of medications was based on the published clinical trial data.

Search Strategy

A systematic search was conducted in the following databases: PubMed, MEDLINE, and Scopus. In order to conduct a thorough systematic search about treatments for PWS, multiple searches were undertaken using the following terms: “Prader-Willi Syndrome AND/OR treatment” “Prader-Willi Syndrome AND/OR medication treatment” “Prader-Willi Syndrome AND/OR growth hormone” “Prader-Willi Syndrome AND/OR oxytocin” “Prader-Willi Syndrome AND/OR Metformin” “Prader-Willi Syndrome AND/OR GLP-1 Receptor Agonist” “prader-Willi Syndrome AND/OR Exenatide” “Prader-Willi Syndrome AND/OR Beloranib” “Prader-Willi Syndrome AND/OR Liraglutide” “Prader-Willi Syndrome AND/OR behavior treatment” “Soles of the Feet”.

1139 articles were identified after the search. After duplication removal and screening the articles with inclusion and exclusion criteria, 34 articles were included in this systematic review. (**Figure 1**)

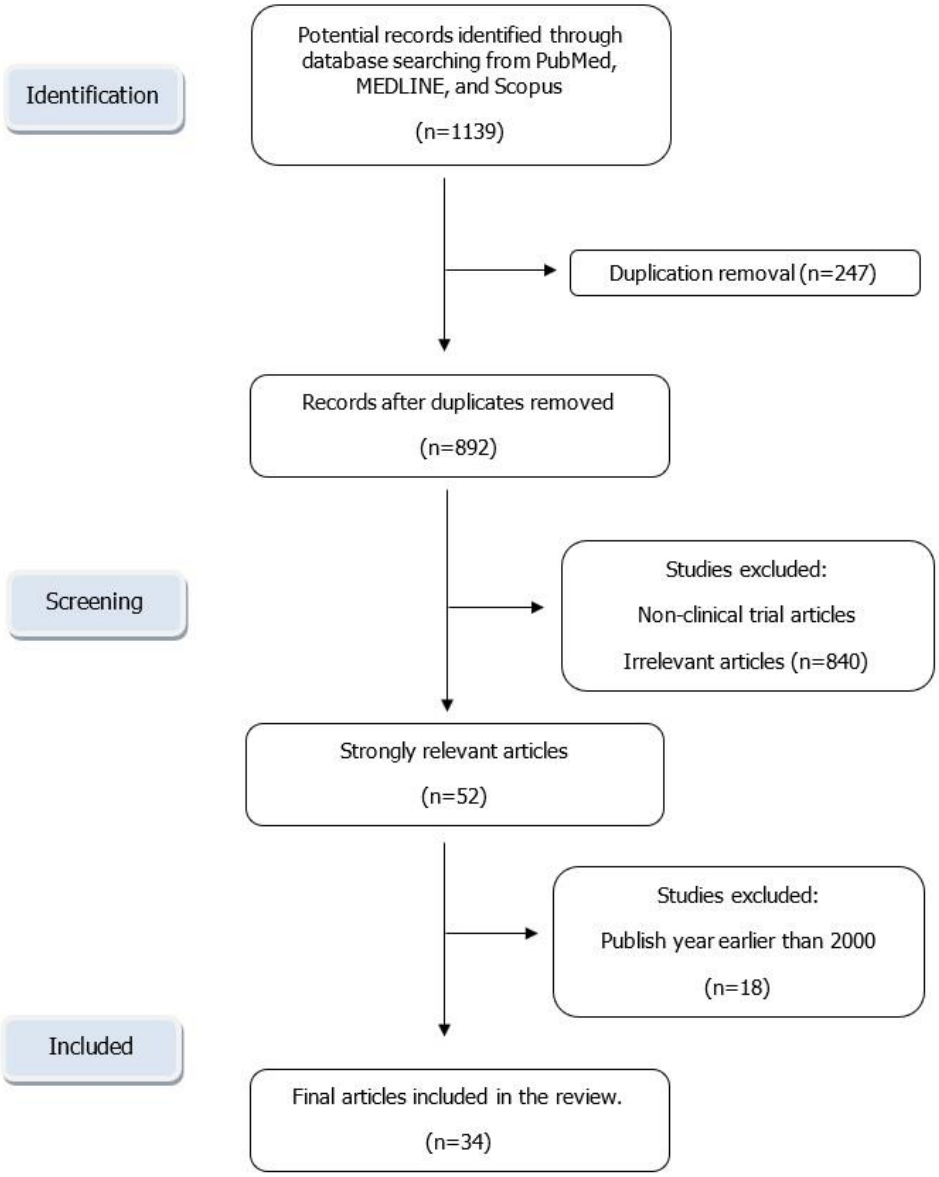

Figure 1. Flow diagram of search strategy and study selection.

Inclusion and Exclusion Criteria

Articles were included when they were: 1) English-written clinical trials (including nonrandomized open trials and randomized controlled trials); 2) studied the efficacy or effect of one or multiple treatments for PWS; and 3) published between January of 2000 and October of 2017. Studies published before 2000 or not strongly related to PWS treatments were excluded.

RESULTS

Based on the above inclusion and exclusion criteria, our final search selected 34 articles. Among these articles, twenty were reports of clinical trials studying the effect of medication treatments for PWS, whose details were showed in three tables (**Table 1-5**). The other thirteen articles focused on other treatment aspects and one focused on the stress level of caregivers, which will be presented in the following discussion section.

Growth Hormone (GH) Studies

GH is the well-established medication for PWS. We selected nine clinical trials focusing on GH after filtration.[6-14] (**Table 1, 2**)

Table 1. Chart of Clinical Trials for Growth Hormone Treatment (Characteristics).

Author(s)	Publication Year	Country of Study	Study Design	Case Number		Age Group	Length	Outcome Measure	Age Group
Bakker Et al.[6]	2017	The Netherlands	Worldwide retros-pective cohort study	Efficacy evaluation	522	4.36 ± 2.88	≥ 3 yr		
				Safety analysis	2332	6.0 ± 4.33	≥ 2 yr before puberty and until adult height	1. heightbody,BMI SDS 2. occurrence of serious adverse events 3. deaths reported in KIGS.	4.36 ± 2.88
Lecka-Ambroziak Et al.[7]	2017	Poland	Case control study	36		7.98	-	1. nasal respiratory flow (by PSG) 2. respiratory effort 3. blood oxygen saturation	7.98
Kupens Et al.[8]	2016	The Netherlands	Randomized, double-blind, placebo-controlled crossover study	27		17.2	2 years	1. blood pressure 2. TC,LDLc and HDLc 3. triglyceride (TG) 4. GF-I,IGFBP-3, and OGTT	17.2
Butler Et al.[9]	2013	U.S.	One-Group Designs	11		32	2 years	1. Electrolytes, IGF-I, glucose, thyroid, insulin, lipids. 2. body composition 3. physical activity and strength 4. diet, energy expenditure and quality of life data	32
Sode-Carlsen Et al.[10]	2010	Denmark	Randomized, double-blind, placebo-controlled study	46		28.7	18 months	Body composition (measured by computed tomography and dual-energy x-ray absorptiometry)	28.7
Carrel Et al.[11]	2010	U.S.	Observational study	48		5–9	-	1. Percent body fat 2. lean body mass 3. carbohydrate/lipid metabolism 4. motor strength (compared using analysis of covarianc)	5–9
Gondoni Et al.[12]	2008	Italy	One-Group Designs	12		26.4±4.4	12 months	1. Body composition (measured by Dual Energy X-ray Absorptiometry) 2. physical performance (evaluated using treadmill exercise test)	26.4±4.4
HoybyeEt al.[13]	2007	Sweden	Cohort study	14		median age 31	6 Years	Body composition (measured by Dual Energy X-ray absorptiometry)	Body composition (measured by Dual Energy X-ray absorptiometry)
Hoybye Et al.[14]	2003	Sweden	Randomized placebo-controlled clinical trial	19		17-32	2 years	1. Body composition (using dual energy X-ray absorptiometry) 2. metabolic and endocrinological parameters (OGTT)	1. Body composition (using dual energy X-ray absorptiometry) 2. metabolic and endocrinological parameters (OGTT)

Table 2. Chart of Clinical Trials for Growth Hormone Treatment (Results).

Authors	Results							
	Height	Lean body mass	Body fat	Appetite	BMI	LDL	HDL	Others
Bakker Et al.[6]	completely normalized	-	-	-	increased	-	-	-
	increase 0.95 SDS	-	-	-	No change	-	-	No increase in mortality rate
Lecka-Ambroziak Et al.[7]	-	-	-	-	-	-	-	oxygen desaturation index increased in short-term group
Kupens, Et al.[8]	-	-	-	-	-	No change	No change	increased fasting glucose and insulin
Butler, Et al.[9]	-	increased	decreased	-	-	-	increased	1.increased IGF-I levels and improved energy expenditure
Sode-Carlsen Et al.[10]	-	increased	decreased	-	-	-	-	increased IGF-I
Carrel, Et al.[11]	increased	-	decreased	-	-	decreased	increased	greater motor strength and sit-ups
Gondoni, Et al.[12]	-	increased	decreased	-	-	-	-	Attained metabolic equivalents improved
Hoybye, Et al.[13]	-	increased	non-significantly decreased of 5%	-	-	-	-	-
Hoybye, Et al.[14]	-	increased	decreased	-	-	-	-	body composition changes in PWS genotype.
Bakker, Et al.[6]	completely normalized	-	-	-	increased	-	-	-
	increase 0.95 SDS	-	-	-	No change	-	-	No increase in mortality rate
Lecka-Ambroziak Et al.[7]	-	-	-	-	-	-	-	oxygen desaturation index increased in short-term group
Kupens Et al.[8]	-	-	-	-	-	No change	No change	increased fasting glucose and insulin

Among these nine studies, six were published between 2010 and 2017, and the other three were published between 2000 and 2010. These studies focused on the effects of GH treatment on the body composition and metabolic index of affected individuals with PWS. In six out of nine studies, patients have either reduced body fat percent or increased lean body mass significantly, covered the age group from age 5 to 30s, treatment time from 12 months to 6 years, two of them were randomized trials.[9-14] Among these trials, there is a large-scale retrospective cohort study, average age 4-6, with treatments of 3 years or longer. It reports significant height improvement after the treatment.[6] Greater motor strength was reported in one study.[11] Lipid profile HDL and LDL were tested in three trials, and two of them showed decreased LDL and increased HDL.[8,9,11] These studies showed no significant adverse effect or safety issue with the treatments except transient blood sugar elevation in one study, and 12 deaths out of 2332 safety analysis in the retrospective cohort, which was lower than the reported 3% annual mortality rate in patients with PWS.[6]

Oxytocin (OXT) Studies

Four studies on OXT published between 2011 and 2017 were selected. (**Table 3**)

Effects of OXT treatment on the behaviors of patients with PWS were mainly investigated. Three of the studies showed behavioral improvements after OXT treatment, which included less food-related behavior, decreased sadness and anger tendencies, increased trust in others and improved social behaviors. However, one out of the four studies showed no significant change.[17] There was only one study showed a modest increase in height after treatment.16 Meanwhile, none of those studies showed any significant changes in BMI, even though a reported appetite decrease in two studies.[15,16]

Table 3. Chart of Clinical Trials for Oxytocin.

Author(s)		**Miller Et al.[15]**	**Kuppens Et al.[16]**	
Publication Year		2017	2016	
Country of Study		U.S.	The Netherlands	
Study Design		Double-blind, placebo-controlled, crossover study	Randomized, double-blind, controlled crossover trial	
Age Group		5 Years to 11 Years	6 to 11 years	11 to 14 years
Case Number		24	17	8
Length		6 weeks	4 weeks	
Outcome Measure		1. Questionnaires 2. blood testing	1. Height, BMI, percentage fat (measured by DXA)	
Results	Height	-	increased	increased
	BMI	-	No significant change	No significant change
	behaviroal parameters	improved socialization, anxiety, and repetitive behaviors	improved anger, sadness and conflicts	happiness, anger and sadness were negatively influenced
	Appetite	decreased	decreased	no changes
	Others	1.All scales factor improvement from Day 3 to Day 6 favored oxytocin over placebo. 2.No single factor showed a statistically significant difference between groups at Day 6.	-	-

Author(s)		**Einfeld Et al.[17]**	**Tauber Et al.[18]**	
Publication Year		2014	2011	
Country of Study		Australia	France	
Study Design		Double-blind randomized controlled trial	Double-blind, randomised, placebo-controlled study	
Age Group		12-30 years	18.7 to 43.6 years with median age 28.5	
Case Number		30	24	
Length		18 weeks	-	
Outcome Measure		Questionaires	Behaviours (scored by psychologis using an in-house grid)	
Results	Height	-	-	
	BMI	-	-	
	behaviroal parameters	increased trust decreased sadness,disruptive behaviour,less conflict -	increased trust decreased sadness,disruptive behaviour,less conflict -	happiness, anger and sadness were negatively influenced
	Appetite	no changes	-	
	Others	-	-	

Table 4. Chart of Clinical Trials for Diabetes and Obesity Drugs (Characteristics).

Authors	Publication Year	Country of Study	Medication	Study Design	Case Number	Age Group	Length	Outcome Measure
Salehi Et al.[19]	2017	U.S.	Exenatide	Open-label, non-randomized, longitudinal, single group assignment study	10	13-25	6 months	1. Weight, BMI 2. truncal fat 3. appetite 4. plasma acylated ghrelin
Sze Et al.[20]	2011	Australia	Exenatide	Single-blinded, randomized, crossover design study	19	30.8	2 weeks	1. Glucose, insulin, glucagon 2. peptide YY, glucagon-like peptide-1, ghrelin 3. appetite 4. energy expenditure
Fintini Et al.[21]	2014	Italy	GLP-1 Receptor Agonist (Liraglutide/Exenatide)	Case report	6	20.7–37.7	24 months	1. BMI, waist circumference. 2. metabolic parameters
Senda Et al.[22]	2012	Japan	Liraglutide	Case report	1	25	-	1. plasma levels of insulin and eptin,adiponectin (high molecular), GLP-1 (active) and ghrelin (active) levels 2. visceral fat and subcutaneous fat(measured by CT)
Cyganek Et al.[23]	2011	Poland	Liraglutide	Case report	1	18	14 weeks	1. HbA1c level 2. weight,fat issue amount 3. waist circumference.
Miller Et al.[24]	2014	U.S.	Metformin	Pilot, open-label study	31	11.18	3 months	1. Body fat (measured by dual-energy X-ray absorptiometry) 2. BMI SDS 3. appetite and satiety (using Hyperphagia Questionnaire)
Authors	Publication Year	Country of Study	Medication	Study Design	Case Number	Age Group	Length	Outcome Measure

Table 5. Chart of Clinical Trials for Diabetes and Obesity Drugs (Results).

Authors	Results								
	Height	BMI	Body fat	Appetite	HbA1c	Blood sugar	Lipid	ghrelin	Others
Salehi Et al.[19]	-	No change	No change	decreased	decreased	-	-	No change	-
Sze Et al.[20]	-	-	-	decreased	-	decreased	-	No change	1. lowered insulin, increased insulin secretion rate 2. decreased PYY and glucagon-like peptide-1 3. Unchanged energy expenditure
Fintini Et al.[21]	-	decreased	-	-	decreased	decreased	-	-	decreased waist circumference
Senda Et al.[22]	-	decreased	decreased	decreased	decreased	-	-	decreased	1. decreased Leptin 2. increased Insulin 3. Normal range of leptin and adiponectin level
Cyganek Et al.[23]	-	decreased	decreased	-	decreased	-	-	-	1. decreased waist circumference 2. slightly increased Fasting C-peptide and insulin levels
Miller Et al.[24]	-	decreased	-	decreased	-	-	-	-	Responders to metformin had higher 2-h glucose levels on OGTT and higher fasting insulin levels.
Kim Et al.[25]	-	dose-dependently decreased	decreased	decreased	-	-	decreased Triglycerides	-	Changes in Behavior were consistent with dose-dependent changes in the Total score

Studies of Medications for Diabetes and Obesity

One study on Beloranib, five studies on Liraglutide or Exenatide and one study on Metformin were included.[19-25] (**Table 4, 5**)

All of these studies focus on the effects of diabetes and obesity drugs on patients with PWS. After 4 weeks' Beloranib treatment in 17 adult patients, body weight, and body mass reduction were detected along with improved biochemical indexes including triglycerides, the decrease in BMI was dose-dependent.[25] As for the five studies focusing on GLP-1 receptor agonists, results showed that patients had decreased appetite and reduced BMI in three trials, decreased body fat and waist circumference in two trials, and mostly with reduced HbA1c level after treatments.[19-23] The Metformin study involved 31 patients with treatment of 3 months, showed reduced appetite and BMI, and blood sugar level.[24] None of seven trials included height as a measurement. No significant adverse effect was observed in these studies.

Intranasal Oxytocin (OXT)

As an emerging focus area for researchers, OXT has been considered a promising medication for PWS. Most characteristics of PWS result from the absence of expression of the paternally derived genes located on chromosome 15 at the locus q11·2-13, and one of the not expressed genes in this region is MAGEL2, whose deficiency might lead to a major reduction of OXT.[29] OXT has an important role in influencing the life quality of patients with PWS, such as feeding behaviors, social interactions, and emotional reactivity.[15] Most of our selected studies confirmed the improvement in behavior after OXT treatment. However, there was one study by Einfeld failed to show any impact.[17] We believe that this could be related to multiple factors. First, OXT works better in younger children. In the study of Kuppens et al, significant changes in behaviors after OXT treatment were only reported in the group of children under 11 years old, whereas subjects in Einfeld's study were all over 12 years old. Secondly, the delivery of OXT may also be a concern. In Einfeld's study, there was no plasma analysis, therefore it was hard to determine whether OXT was successfully delivered.

Although some results indicated increase in height and decrease in appetite with OXT treatment, the data is very limited this point. To validate OXT's effect on body mass and height, further studies with longer duration and bigger sample size are needed, set both body fat composition and lean body mass as well as BMI, metabolic profile as outcome measurements, in addition the efficacy of delivery and bioavailability of intranasal oxytocin will also need to be studied. Furthermore, the data of OXT effect on behaviors is far from enough, the future studies should focus on OXT treatment on social communication and behavioral profiles cross different age groups, and different severity or co-morbidity subgroups. We foresee extending clinical usage with more data collected.

Drugs for diabetes mellitus and obesity

As pointed out above, some drugs for diabetes mellitus also could potentially play an important role in the treatment of PWS.

Metformin is the first line treatment for Type 2 Diabetes Mellitus (T2DM), with the main effect to decrease liver glucose production. Researchers currently relate this medication to the treatment of patients with PWS since T2DM and PWS share a lot of similarities and connections. With its known weight loss capacity, we believe that Metformin has the potential to be an important part of treatment for PWS especially those associated glucose intolerance and insulin resistance. More studies with larger scale are needed to confirm the efficacy, the best time to initiate, with or without diabetes.

GLP-1 receptor agonists started to catch the attention as well. Multiple studies have been conducted to investigate the effectiveness of GLP-1 agonist treatment in patients with PWS, whose results supported the efficacy in different levels.[19-23] In view of its weight loss benefit and anti-diabetes effect, similar to metformin, there should be some value of this group of medications in the treatment of PWS, particularly those with glucose intolerance or diabetes. Further studies are necessary to investigate the long-term effects of GLP-1 receptor agonists on body mass and composition in PWS, as well as their adverse event profile. Their administration route as an injection might limit its use.

Apart from Diabetes medications, there is another noteworthy drug called Beloranib, an inhibitor of the enzyme METAP2, is a former drug candidate to treat obesity. It has approved efficacy in body weight loss and hyperphagia reduction, which are very important for PWS. Beloranib should have some potential to serve as a medication for patients with PWS, however this medication halted during phase III clinical trail due to unclear second deaths, further studies need to be conducted to assure the safety before it could move forward.

Other treatment perspectives

We discussed three major drug categories in PWS, there are other important aspects of treatments should be addressed here. First of all, early detection and intervention are paramount and currently still lag behind.[30,31] Prenatal screening methods including DNA methylation and high-resolution chromosomal SNP microarrays should be considered and newborn screening (NBS) could be applied basing on the utilization of next-generation sequencing and focusing on multiple PCR-based fragments from copy-number-determining chromosomal regions.[32] Early detection and early intervention lead a much better prognosis.

Secondly, caregivers play a very important role[33] in PWS treatment, however, most parents suffer from significant stress and require a great deal of counseling and training.[34] Their stress level and parenting style directly impact on the outcome. Haig and Woodcock investigated 10 caregivers of

patients with PWS through interviews and questionnaires, suggested the importance for caregivers to increase their flexibility and assure smooth transition.[35]

Third, behavioral intervention has been essential for PWS treatment. Patients with PWS share a lot in common in social-cognitive challenges with ASD patients, whose most empirically studied and validated treatment is behavioral treatment.[36] Dimitropoulos, Zyga, and Russ directly delivered a 6-week play-based intervention to eight children through telehealth[37] the participants completed the program without much difficulty and showed good acceptability to the behavioral intervention. Adolescents with PWS usually show aggressive reactions.[38] Soles of the Feet (SoF) is a mindfulness-based meditation technique developed by ONE Research Institute.[39] It kept away from the situations which cause anger and aggressive reactions. To evaluate the effectiveness of this meditation on patients with PWS, Singh, Lancioni, and Myers et al[40] found patients' physical aggression was almost completely resolved and verbal aggression significantly decreased after SoF, and these improvements were maintained after 12 months.

CONCLUSION AND FUTURE DIRECTIONS

This systematic review discussed the latest clinical trials for PWS and discussed the current treatment advancement. Of 1,139 potentially relevant articles from PubMed, MEDLINE, and Scopus from 2000 to 2017, we extracted 34 relevant articles with 20 clinical trials. As discussed above, in addition to the well-established therapy GH with further confirmed efficacy, the newer experimental OXT and diabetes and obesity drugs (Metformin, GLP-1 agonists, and Beloranib) have demonstrated certain positive effect on improving the symptoms of PWS which worth further investigations. Concomitant application among these drugs is also potentially important since they have divergent and complementary functions. Further studies particularly for these new emerging drugs, might also include GH too, are needed to look into their correlation with different PWS subtypes, such as those with different genetic or phenotypes, those co-existed with ASD, Depression, DM, GI disturbances, hypothyroidism or others, correlation of behavioral changes and hormonal parameters, the molecular mechanism of these therapies, and more specific target solutions. The future direction we believe should be, detect early and treat early with effective drug therapies in the combination of behavioral interventions for different subtypes, establish effective protocols, ultimately serve PWS population for better outcomes.

CONFLICT OF INTEREST
None

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Review

ASD and Sleep Disorders

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Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by social communication deficits and repetitive behaviors/limited interests. There have been accumulated reports of significant sleep problems in ASD. The most common sleep problems include difficulties in sleep initiation and maintenance, irregular sleep- awakening rhythm, and disordered sleep pattern. Some investigators have suggested that sleep problems in children with ASD may be due to abnormal circadian rhythm. Neuroendocrine markers provided another perspective to study biological clock, these biomarkers are nearly not affected by social domains, such as cortisol and melatonin levels in ASD. Many sleep related genes are associated with ASD, especially single nucleotide polymorphisms in core circadian clock genes have been convinced the linkage. The abnormal expression of key genes causes alteration of protein synthesis in some critical pathways associated with ASD. Effective sleep therapy is critical to the improvement of the core symptoms of ASD.
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Key Words: *autism spectrum disorder, sleep disorder, biological clock, circadian rhythm, melatonin, cortisol, sleep apnea, sleep related genes*

INTRODUCTION

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by social communication deficits and repetitive behaviors/limited interests.[1] Parental-based surveys showed that the prevalence of sleep disorders is about 44%-83%.[2-5] While in typically developed infants and preschool children is about 9% to 50%[6,7] The sleep disorders in ASD are mostly manifested as difficulty in falling asleep and awakening during sleep, which in turn affects the behavior of ASD individuals. Sleep disorders greatly reduce the quality of life of ASD individuals and their families. The sleep study in children with ASD is becoming a hot topic in ASD and sleep research fields. This review article summarizes the most recent clinical and basic research advances of ASD and sleep disorders.

Characteristics of Sleep Disorders in Patients with ASD and Their Relationship with Behavioral Issues

There have been accumulated reports of most common sleep problems in ASD over the past three decades. In recent years, the number and quality of related reports are significantly increased. The incidence of sleep disorders in ASD is considerably higher than normally developed children, and also higher than those with other developmental disorders.

The most common sleep problems in children with ASD are sleep initiation difficulties, sleep maintenance problems, irregular sleep-awakening rhythm, and disordered sleep pattern[6,8] A sleep questionnaire from Japan[9] assessed 965 cases of normal preschool children and 193 ASD preschoolers, 107 from ASD group were evaluated for the behavioral problems. The results showed that ASD children had significant sleep problems compared with normal preschool children. The further analysis showed that the sleep disorders in ASD are mainly falling into the following categories: insomnia including problems of falling sleep and frequent night awakening; waking up crying; irregular sleep rhythm; parasomnia; sleep disordered breathing or obstructive sleep apnea, daytime sleepiness.

ASD children have a lot of behavioral problems, including physical aggression, hostility, inattention, hyper-responsiveness, irritability, and hyperactivity. ASD children with sleep problems show more behavioral problems than children who do not have sleep problems. The severity of sleep problems, especially the severity of insomnia, is highly correlated with the behavioral problems they have. Several studies in ASD children describe the relationship between sleep deprivation and behavioral affective disorders, which can present as hyperactivity, mood instability, worsen aggression, emotional abnormalities,[10] behavioral problems

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and poor adaptive skill development,[11] in addition, preliminary studies showed lack of sleep correlates with nonverbal intelligence defects,[12] reduced communication skills[13] and academic performance.

As stated above, sleep problems are well recognized in ASD children, especially obstructive sleep disorders are common in preschool children with ASD. Current studies suggested that the sleep problems, particularly insomnia, are associated with behavioral problems in ASD preschool children, which highly suggested that routine assessment and treatment of sleep problems should be greatly beneficial to autistic children and their families.

CHANGES OF BIOLOGICAL CLOCK IN ASD

The biological clock is also referred as circadian rhythm, the human sleep-wake cycle, the body's inner clock, a biological process that displays an oscillation of about 24 hours. Most recently, Jeffrey C. Hall, Michael Rosbash, Michael W. Young share won 2017 Nobel prize for their discoveries of molecular mechanisms controlling the circadian rhythm.[14]

Some investigators have suggested that sleep problems in children with ASD may be due to abnormal circadian rhythm.[15] Previously the clear association between sleep disorders and circadian rhythm disturbances in ASD children had been rarely reported.[16,17] More studies have been published in recent years.

Sleep duration was more a focus of the studies, which indicated its association with ASD. Veatch and colleague found sleep duration negatively correlated with the severity of ASD core symptoms, and positively correlated with IQ scores [18] Limoges and colleague[19] illustrated that the shorter sleep duration is associated with social impairment and comorbidities in ASD. This study indicated a significant negative correlation between slow-wave sleep (SWS) and learning capacity of a sensory-motor procedural memory task. Another ASD study involved 5-16-year-old male patients [20] found that the total sleep time of ASD individuals was significantly less than the control group. A study[21] with ASD individuals aged 12 to 24, reported more reduced effective sleep time and increased night awakening in autistic patients than the normal controls.

Other ASD sleep disturbances include difficulty to fall in sleep; frequent night awakening, lower sleep efficiency (sleep fragmentation). Wiggs and colleagues[22] confirmed that autistic children have more prolonged incubation periods, delayed or advanced sleep phases, and increased night awakening, which were consistent with the previous findings from sleep diaries and questionnaires. These patterns of sleep abnormalities are very similar to those circadian rhythmic sleep disorders described above. The study showed that eight children were identified as biological clock sleep awakening problems, which could be good representations of the biological clock sleep disorders. The other studies in children with ASD, also indicated the longer sleep latency,[23,24] frequent night awakenings,[25] lower sleep efficiency (e.g., sleep time and bedtime ratio),[26] reduced non-REM and SWA sleep, lower sleep spindle density, REM sleep abnormality, periodic limb movement during sleep, decreased the first two thirds of the sleep time.[27-30] Another study involved 21 ASD patients aged 4-10 years old, used the more strict inclusion criteria[31] excluded those with mental retardation, seizures, and drug use, still showed reduced sleep efficiency, delayed sleep latency, and reported as "poor sleepers" by parents. The early stage of the SWA reduction is a sign of the weakening of steady-state sleep function.

The study methods of circadian rhythm developed with the advancement of physical technique.

The wrist actigraphy and polysomnography have more advantage than parental subjective reporting and sleep diaries. Several studies used objective tests have confirmed the findings from sleep diaries and questionnaires. Particularly, polysomnography has been used as a more reliable method of studying sleep structures under relatively controlled conditions.

The previous work mainly focused on the sleep disturbance of ASD individuals, most commonly reported above problems indicated an involvement of the biological clock system, although it seems that the irregular sleep mode initiated by the biological clock is only part of the problems. This subtype also represents a relatively large portion of the previous reports. The future work may focus on circadian rhythm gene and protein expression, as mentioned above.

HORMONE CHANGES ASSOCIATED WITH BIOLOGICAL CLOCK ALTERATIONS IN ASD

There have been reported studies of melatonin as well as cortisol levels in autistic children using blood, urine and saliva specimens. These studies provided a better understanding of alterations of biological clocks in children with ASD.

MELATONIN

Melatonin is produced in the dark by the pineal gland and is a key regulator of circadian and seasonal rhythms.[32] A lower melatonin level has been reported in individuals with ASD.

Chamberlain and Herman[33] first noted that melatonin secretion was abnormal in children with ASD in 1990, suggesting that there was a high secretion status of this hormone in a subgroup of these children, while the subsequent studies [34-36] showed problems of producing Melatonin in ASD.

Two studies [34,36] have found that the magnitude of melatonin rhythm is generally reduced, and the level is decreased in the nighttime. Kulman[34] reported 14 cases of autistic patients not only had a lower average level of melatonin at night, but also showed abnormal melatonin rhythm comparing with control group. In particular, most autistic patients showed a decrease in the gap between daytime and nighttime melatonin levels, one of the smaller subgroups showed a reversal of the

circadian rhythm, which can also be observed in Smith-Magenis syndrome (SMS).[37] Tordjmanand his colleagues [38,39] conducted a larger controlled study and got similar results. Autistic children showed abnormal nighttime 6-sulpho melatonin levels. 63% of autistic children has less than half of 6-sulphated melatonin levels compared with the mean of the control group, the night time 6-sulphated melatonin levels were found to be negatively correlated with severity of autistic impairments in verbal communication and play.[38,50]

Nir and colleagues[36] have found that older autistic (26-30 years) patients do have a tendency of increased melatonin at night. Other study[35] found that most autistic patients had lower plasma melatonin levels in the early morning. This significant inheritance may be due to mutations in the potential genetic component ASMT, which encodes an enzyme that affects melatonin synthesis.[35]

CORTISOL

Cortisol is a corticosteroid hormone found in humans, there are variations different times of the day.[40,41] The peak level of cortisol is in the morning after awakening, stays a while then rapidly declines, the rate of the reduction will slow down in the afternoon, reach the lowest level in the evening.[40,42,43] The studies of cortisol levels and rhythms in children with ASD showed mixed results, because there may be the potential confounding effects of hormones under stress. The blood draw for cortisol studies itself could be a stress and also may contain more influential factors.[44] In order to minimize the potential impact caused by the stress, most of the laboratory studies of cortisol are using saliva [42,43] or urine specimens rather than blood samples, thus the collection can be carried out at home, this case the patient doesn't to enter the external environment, and the stress should be minimal.

Corbett and his colleagues[45] reported that the ASD group's peak-to-trough cortisol level was different from the control group. Results showed abnormal daytime fluctuations in autistic individuals. Hill's study[46] indicated relatively advanced cortisol peak level, reduced overall daytime level and multiple peaks in ASD group. However, Richdale and Prior[47] implied that increased cortisol in ASD could be related to stress. Interestingly, Nir's study[36] showed no differences in serum cortisol levels among various ASD groups compared with control. Goldman's results[48] also showed no difference of salivary cortisol between ASD and control in adolescents/young adults, although they compared the morning cortisol, evening cortisol, and the morning-evening difference between two groups.

As mentioned above, there are special challenges to check the hormone level by collecting a variety of samples within 24 hours, particularly for ASD individuals with low tolerability. Besides, there are individual differences, broad-spectrum functional deficiencies, which constitute the heterogeneity of ASD and sleep behavior. In addition, differences in methodologies and analytical methods can also partly explain the inconsistency of the results, especially when the differences in assay methods and the collection techniques (such as saliva, blood, etc.) can lead to sensitivity to changes in the measured hormones. This is a major problem to study the overall profile of melatonin and cortisol particularly cortisol. It may be helpful to evaluate cortisol levels over a few days to obtain the overall picture of its biorhythm. It's worth mention that, Melatonin can be strongly inhibited by light32, further research must include strict control of lighting and photometric determination.

CHANGES IN SLEEP-RELATED GENES AND PROTEIN SYNTHESIS IN ASD

Many sleep-related genes are associated with ASD, especially single nucleotide polymorphisms in core circadian clock.[49] There are twenty-three genes involved in ten biological Circadian rhythms, which are associated ASD.[50]

Many genes (ATP13A4, CDH9, CDH13, CNTNAP2, CTNNA3, DIAPH3, GRIN2A, MDGA2, NLGN3, NLGN4, NRXN1, SHANK3 et, al) have been associated with ASD.[51] Genetic studies revealed many genes encoding synaptic proteins are associated with susceptibility to ASD, which includes genes NLGN3, NLGN4, and NRXN1 encoding the synaptic cell adhesion molecules and SHANK3 encoding a postsynaptic scaffolding protein. This protein complex is crucial for the maintenance of functional synapses as well as the adequate balance between neuronal excitation and inhibition.[52] Sarowar T, et al [53] found that Circadian rhythms may be able to modulate Shank3 signaling and then synaptic function. The expression of Shank3alpha increases rapidly by induced activity in thalamus and cortex. In the hippocampus, changes in synaptic Shank3 expression levels are influenced by circadian rhythm/melatonin concentration, while running activity increases Shank[3] expression in the cortex and decreases its expression in the striatum.[53]

Veatch et al found out that sleep onset delay relates to melatonin pathway genes.[54] They observed that decreased ASMT expression and related to decreased CYP1A2 enzyme activity. There is a relationship between genotypes in ASMT and CYP1A2. A recent study suggested that functional defects from NR1D1 may be related to ASD pathogenesis.[55] Nr1d1 was found to play a pivotal role in corticogenesis via regulation of excitatory neuron migration and synaptic network formation. Mutations in ASMT gene, encoding the last enzyme of the melatonin pathway have been reported as a risk factor for ASD.[56]

Diaz-Beltran L, et al identified a set of 19 genes not previously linked to ASD that were significantly differentially regulated in individuals with ASD. These genes were of potential etiologic relevance to ASD, given their critical roles in neurological processes crucial for optimal brain development and function, learning and memory, cognition and social behavior.[57] A recent study showed that there is a significant association between rs7794745 CNTNAP2 gene polymorphism and ASD in the studied population.[58]

ASD behavior subtypes may represent different biological phenotypes. The resulting gene expression profiles distinguish between ASD subtypes, which correlates the "biotype" and the behavior or symptom.

The treatment of sleep disorders should focus on the abnormal expression of key genes. For example, aryl-alkylamine N-acetyl transferase (AANAT) is a rate-limiting enzyme in the process of melatonin synthesis. It is down-regulated in this subtype of ASD. The enzymatic mechanism for melatonin deficit in ASD, involving a reduction of the enzyme activities contributing to melatonin synthesis (AANAT and ASMT), was observed in the pineal gland as well as in gut and platelets of patients.[59] This finding suggested that melatonin supplementation can improve the circadian rhythm and relevant neurological function.

In fact, the synaptic function and its relation to the biological clock were previously proposed. Another possible factor within the network with therapeutic potential is dihydropyrimidine dehydrogenase (DPYD). Lacking enzymes produced by DPYD will cause individuals to suffer from epilepsy and mental retardation, as is the case with ASD60. Due to the high risk of epilepsy and related neurological problems, individuals with ASD who lack DPYD showed to have the greatest sensitivity to antagonize convulsive drugs. In this way, AANAT and DPYD, as disease markers, can serve as potential diagnostic markers for ASD severe subtypes as well as potential therapeutic targets, especially when these enzymes are reduced in affected individuals.

Ca(2)(+)-dependent activator protein for secretion 2 (CAPS2) protein are critical for normal brain development and behavior, and that allelic changes due to copy number variation (CNV) may contribute to autistic symptoms in combination with deficits in other autism-associated genes.[61]

Fragile X syndrome (FXS) is the most common monogenic form of autism spectrum disorder (ASD). FXS results from the loss of fragile X mental retardation (FMR1) gene products, fragile X mental retardation protein (FMRP), which triggers a variety of physiological and behavioral abnormalities.[62] This disorder is also correlated with clock components underlying behavioral circadian rhythms and, thus, a mutation of the FMR1 gene can result in disturbed sleep patterns and altered circadian rhythms.

Retinoic acid-related orphan receptor alpha gene (RORa) and the microRNA MIR137 have both recently been identified as novel candidate genes for neuropsychiatric disorders. According to the RORA-deficient staggerer mouse model study, these functions include cerebellar development, differentiation and survival of Purkinje cells,[63] regulation of neuroprotection and circadian rhythm.[64] Devanna and Vernes found the role of MIR137 as an ASD candidate gene and demonstrated a direct biological role of these previously unrelated ASD candidate genes.[65]

The sleep mechanism is well-characterized in zebrafish and key regulators of the sleep/wake cycle are conserved, including melatonin and hypocretin/orexin (Hcrt), whereas novel sleep-regulating proteins, such as Kcnh4a, Neuromedin U, and QRFP, are continually being identified.[66]

More studies [67-69] have found circadian rhythm associated with genes which encode predominantly nuclear protein in adult Drosophila.[70-73] There is little genetic study focus on circadian rhythm and ASD. We believe that it is an attractive field to explore. Genetic study, protein expression and treatment targeting specific genes or proteins associated with ASD circadian rhythms may become a promising research area in the future.

CONCLUSION AND TREATMENT PERSPECTIVE

We discussed the various sleep disorders in ASD and their high correlation, emphasized the biologic clock changes, related biomarkers, genes, and protein synthesis, offered further understanding of molecular mechanism of circadian rhythm. More importantly the effective sleep therapy is critical to the improvement of the core symptom of ASD and the life quality of those affected individuals and their families.[74]

Conducting sleep education and developing appropriate and individualized behavioral therapy strategies are first-line treatments for ASD children with sleep disorders.[75] The drug interventions are considered only when the behavioral treatment is unsuccessful or there is no short-term drug-assisted implementation of behavioral therapy.[76] Some medications approved to treat aggressive or self-injurious behavior, severe mood swings, irritability, such as Risperidone, the serotonin-2 receptor and antagonizes dopamine D2 receptors, which increased daytime sleepiness and insomnia at night as common side effects.[77-79] Selective serotonin reuptake inhibitors (SSRIs) is commonly used to treat repetitive behavior in ASD.[80] Melatonin supplement is increasingly used in the treatment of ASD children, currently proven to be effective in improving sleep.[81-84] It can restore the circadian rhythm of ASD.[85,86] Exogenous melatonin supplementation can also be effective in treating sleep bursal disorders such as sleep phase abnormality, in which case melatonin should be administered at a specific point of time based on the onset or advancement of sleep initiation time.[74] It has been reported that melatonin treatment of insomnia can improve the problem behavior and academic performance of children with Asperger's syndrome.[87] When behavioral therapy and melatonin treatment are ineffective, other medications can be considered including clonidine, mirtazapine, gabapentin.[88,89] Although Risperidone can shorten sleep latency, the side effects are serious, it's not recommended for insomnia alone.[90] Hyperbaric oxygen (HBOT) therapy should not be used for the treatment of ASD.[91]

Sleep apnea (SDB) is common in ASD children, SDB treatment mainly includes ventilator CPAP and surgical intervention, the first-line surgical treatment of children with

OSA is most commonly used tonsillectomy,[92] which has been reported in a 5-year-old ASD Child[10] with obstructive sleep apnea underwent tonsillectomy improved their daytime behavior, while in another 4-year-old child with ASD,[93] successful sleep intervention improved the patient's self-injury behavior and night awakening. Some children require continuous positive airway pressure (CPAP) or additional surgical treatment after tonsillectomy, especially those obese children and those with concealed craniofacial deformities.[94] Other treatments include rapid maxillary dilatation, weight loss postural treatment.[95]

Sleep disorder is highly related to ASD. Obstructive sleep disorders are very common in preschool autistic children. Current studies suggest that sleep problems, especially insomnia, are associated with behavioral problems of ASD preschool children. These results highly suggest that routine assessment and treatment of sleep problems will greatly contribute to autistic children and their families. Early identification and intervention of childhood sleep problems for children with ASD are essential to prevent later negative outcome and complications. In the future, novel drug targets for ASD may have a great advancement based on proteomic studies.[96] Genetic treatment targeting specific genes or proteins associated with ASD circadian rhythms may become a promising research area.

CONFLICT OF INTEREST

None.

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Review

Efficacy of Mindfulness- and Acceptance-Based Treatments for Culturally and Linguistically Diverse Patients: Communicating This to Patients

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There is a growing consensus that the use of mindfulness- and acceptance-based behavioral therapies can result in physiological and psychological benefits in Clinical and non-Clinical populations. However, the research on the use of such therapeutic approaches with culturally and linguistically diverse (CLD) populations is still in its infancy. This article reviews the efficacy of mindfulness- and acceptance-based treatments in terms of physiological outcomes, as well as the evidence thus far for their efficacy with CLD groups. We also provide suggestions for communicating with CLD patients about the potential benefits of mindfulness- and acceptance-based approaches in the treatment of stress-related conditions.
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Key Words: *mindfulness- and acceptance-based treatments, efficacy, cultural and linguistically diverse populations.*

INTRODUCTION

Although mindfulness- and acceptance-based practices have existed in numerous cultures and for thousands of years,[1,2] it is only within the past few decades that the endocrinology and neurophysiology associated with such practices have been explored. Mindfulness- and acceptance-based therapies (MABTs) or acceptance-based behavioral therapies (ABBTs) broadly refer to therapeutic approaches that seek to alter patients' relationship with seemingly aversive or undesired internal experiences.[3] This perspective argues that avoidant patterns of responding to internal experiences can result in dysfunctional experiential avoidance and distancing from "valued actions" [5] in daily living. The increase in knowledge regarding the biological mechanisms associated with (MABTs) has significant implications in terms of understanding how MABTs work and how to effectively apply them in healthcare. However, in order to effectively translate this new research into meaningful interventions for culturally and linguistically diverse (CLD) patients, a process of cultural consideration becomes necessary. This is based on the research on Cultural Match Theory (CMT), which proposes that as the cultural characteristics of the treatment align with those of the patient, then the effectiveness of the treatment will increase.[5,6,7] This article will review the potential for MABTs in mitigating the negative impacts of chronic stress, as well as make recommendations for having conversations with CLD patients about stress and encouraging the exploration of MABTs as a method of self-care or a low-cost intervention.

The Potentials of MABTs in Managing Stress

Due to overwhelming demands in life and/or work, financial concerns, chronic illness, or traumatic experiences, many of our patients are under prolonged stress. In 2015, Americans reported stress levels of 5.1 on a 10-point scale (wherein 1 = "little or no stress" and 10 = "a great deal of stress"), with 24% of adults reporting levels of stress as 8.9 or higher.[8] Worries concerning money and work were the top two reported stressors respectively.[8] Many CLD patients in particular may experience higher levels of stress, due to acculturation and safety concerns in their communities.[9] They also often encounter cultural and linguistic barriers, acculturative stress, prejudice and discrimination, and limited financial resources.[10,11] Chronic or "toxic" stress[12] has been found to be connected to a myriad of negative health outcomes, such as: headaches, hyperventilation/panic attacks, cardiovascular disease, increases vulnerability to Type-II diabetes, heartburn, diaherra and/or constipation, and sexual difficulties in males and females.[13]

With stressors like money and work likely not disappearing in the foreseeable future, it may be conducive to cultivating better health for people to develop effective coping strategies for managing stress. The learning of mindfulness and acceptance strategies may be one such coping strategy for developing ways of being and doing that allow one live more harmoniously amongst a sea of troubles. Mindfulness has been defined as "awareness that arises through paying attention, on

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purpose, in the present moment, nonjudgmentally."[14] Researchers such as Kabat-Zinn originally began using programs such as Mindfulness-based Stress Reduction (MBSR) to help people with chronic pain who were not responding well to standard medical treatment.[15] Since that time, a plethora of research has been conducted based on Kabat-Zinn's transmutation of mindfulness-meditation from a religious practice to a medical intervention. Specifically regarding stress-related disease, mindfulness-meditation has been found to benefit the treatment of chronic pain,[16] anxiety disorders,[17] depression,[18] and hypertension[19] as a few examples. The cultivation of nonjudgmental/non-elaborative awareness through the practice of mindfulness provides the foundational skills for changing one's relationship with unwanted internal experiences. The importance placed on acceptance in MABTs comes from the belief that the experiential avoidance of aversive internal stimuli (e.g., thinking only 'weak' people get depressed) limits one's cognitive and behavioral repertoire in dealing with inevitable difficulties in life. Experiential avoidance also strengthens the negative valence associated with unwanted stimuli/experience through a process of negative reinforcement (i.e., temporarily relieving suffering, but maintaining in the long-term the cognitive and behavioral processes associated with the unwanted stimuli/experience). What is the hypothesized relationship between the activities associated with MABTs and the healing of the body/mind? MABTs are thought to effectively alter patients' relationship with their internal experiences, which may have been previously restricted, avoided, or were even beyond their awareness.[20] For example, MBSR's emphasis on experiencing bodily sensations without judgment but instead with equanimity, may help people harness the benefits of the "relaxation response"[21] in situations that would typically elicit the 'stress' response. Thus, programs such as MBSR cultivate an 'approach' orientation towards challenges (such as pain) rather than 'avoidance.' With this change in orientation towards potential stressors, systems within the body associated with the relaxation response may be more readily harnessed.

Bodily Responses Associated with the practice of Mindfulness and Acceptance

But how do MABTs access the "wisdom of the body"[22] in the healing process? It appears that the consistent practice of mindfulness and acceptance works synergistically with the neuroplasticity of the brain.[23] Neuroplasticity is a term describing the brain's ability to "reorganize"[23] itself throughout the lifespan through the creation of new neural connections. There is accumulating evidence that the practice of mindfulness and acceptance is 'rewiring' the brain so that the stress response system handed down through evolutionary pressures can be reorganized in a more adaptive fashion towards the pressures of modernity.[23-25]

Two vital parts of the brain associated with the stress response, the prefrontal cortex and the amygdala, appear to be especially prone to reorganization resulting from sustained mindfulness- and acceptance-based training. Experienced meditators have been found using MRI scans to have significantly decreased grey matter volume in their right amygdala and left caudate relative to less experienced meditators.[26] What is more, experienced meditators have also been found to have increased prefrontal cortical thickness relative to less experienced meditators.[27] These findings imply that prolonged training in mindfulness and acceptance may reorganize the brain so that one would have 1) reduced stress reactivity and 2) an increased ability of higher-order attentional and executive functionality. It should be noted that the changes in these neurological pathways correspond with theoretical facets of mindfulness such as non-reactivity and describing.[28]

These structural changes in the brain may explain why experienced meditators are also found to have markers associated with positive psychological and physiological outcomes. For example, experienced meditators were found to have lower levels of c-reactive protein and interleukin 6, which are both pro-inflammatory.[29] The adaptive functioning of experienced meditators may best be seen in the inhibition of the cortisol awakening response (CAR), which has the role of continuing the stress response system in the body if the perceptual system still registers the presence of a threat. Such threats include internal stimuli, such as negative emotions and/or worries. A study examining participants' ability to 1) label and describe inner experiences and 2) accept negative thoughts and feelings without judgement found that such 'dispositional mindfulness' moderated the impact of negative stimuli on the CAR.[30] These authors argue that it is not necessarily the content of one's thoughts, but the way in which one relates to them that moderates the reactivity of the CAR. Moreover, they point out that because the CAR is strongly associated with the experience of waking up, a mindful disposition may be particularly salient in the intensity of hypothalamic-pituitary-adrenal (HPA) axis activity.[30] This is because the process of awakening from sleep is generally paired with a resurfacing of personality characteristics, as well as "anticipation of daily events."[30] Thus, persons with higher dispositional mindfulness may relate to daily events with less anxiety-provoking appraisals, resulting in lower HPA axis activity than those more prone to react strongly to future uncertainties.

Sanada et al[31] conducted a meta-analysis of five studies (many studies were excluded that did not meet the authors' criteria, such as, cortisol could not be collected under stressed conditions, participants had to be healthy individuals, etc.) examining the connection between mindfulness-based interventions (MBIs) and salivary cortisol. Their meta-analysis found a moderately low effect size (ES) (Hedges' g = 0.41), although perhaps most interesting was the moderate heterogeneity found as a result of 1) age, 2) the number of sessions and 3) the total time of the MBI. From the five randomized control studies (RCTs) examined, it appeared that MBIs had stronger effects for younger participants, the greater number of sessions attended, as well as the greater amount of hours spent practicing the MBI. The authors argue that there is evidence that MBIs can have beneficial effects on reducing cortisol levels in healthy individuals, though more rigorous RCTs are needed in order to support these findings.

Need for Further Research on MBIs/MABTs and Stress

As Sanada et al[31] argue, there is a scarcity of rigorously conducted RCTs examining the connection between MBIs and stress reactivity. They argue that cortisol needs to be measured carefully, through considering the time of day of collection, using multiple days of collection, as well as assessing appropriate indicators of cortisol production (e.g., CAR, daily output, and diurnal slope). Sanada et al[31] also point towards the need for the MBIs/MABTs to be conducted using standardized intervention programs, such as MBSR and Mindfulness-based Cognitive Therapy (MBCT). Measures of Treatment Fidelity (i.e., was the program implemented as designed) would also strengthen the link between the 'active ingredients' of the intervention and outcomes (i.e., was it the intervention or something else that led to the outcomes?)

MABTs for Culturally and Linguistically Diverse (CLD) Patients. Efficacy of MABTs for CLD Patients

The research on the acceptability and efficacy of MABTs for CLD patients is still in its infancy. It is noteworthy that a meta-analysis[32] of 32 studies totaling 2,198 patients from nondominant cultural and/or marginalized backgrounds (including racial minorities, refugees, individuals with disabilities and/or low-income) indicated small (Hedges' g = .38) to large (Hedges' g = 1.32) effect sizes for MABTs, which varied by study design. Overall, the studies that examined the efficacy of MABTs for specific minority groups suggest the approach is acceptable and effective. For example, Roth and Creaser[33] evaluated a bilingual MBSR program in an inner-city setting. The program included practices of breathing meditation, eating meditation, walking meditation, and mindful yoga. The authors examined compliance, medical and psychological symptom reduction, and changes in self-esteem, of English- and Spanish-speaking patients who completed the 8-week Stress Reduction and Relaxation Program at a Community Health Center. Results revealed statistically significant decreases in medical and psychological symptoms and improvements in self-esteem. Participants reported dramatic changes in attitudes, beliefs, habits, and behaviors. The findings suggest that a MBSR course can be an effective health care intervention when utilized by English-and Spanish-speaking patients in an inner-city community health center.

Hinton, Pich, Hofmann, and Otto's study[34] with traumatized refugees and ethnic minority populations also indicates that MABTs are effective for these populations. They concluded that mindfulness strategies are therapeutic for refugees and minority populations because mindfulness increases their psychological flexibility, as well as decreases somatic distress and rumination. They argue that mindfulness functions as an emotion regulation technique that decreases the attentional bias to threat and forms part of a new adaptive processing mode. However, Luy's study[35] on the efficacy of mindfulness-based (MB) group counseling with 66 Southeast Asian refugees at a mental health clinic yielded mixed results. She found that the MB group counseling improved global functioning (measured by the Global Assessment of Functioning scale) but not functional impairment (measured by the Sheehan Disability Scale). It is not clear why the MB group counseling did not improve functional impairment for this population. Further study is needed for an answer to this question.

Future research should also examine the potential contraindicative elements of MBIs. There may be certain cultural and clinical populations wherein the use of MBIs may actually increase stress reactivity rather than reduce it. For some, the thought that one could remain fully 'nonjudgmental' may feel like a cognitive impossibility, and thus they may not be as receptive to some current conceptualizations of mindfulness.

Communicating with CLD Patients about Using MABT to Reduce Stress

Because culture influences the ontology and phenomenology of stress,[36,37] it is vital to let patients describe not only the sources of their stress, but their thoughts, feelings, and experienced physiology associated with stress. For example, some Chinese patients might attribute their heart palpitations to having a *xin xu* ("weak heart") and they may focus more on the influence of their diet rather than stress levels on their heart health. In such a case, it might be helpful to highlight the science behind MABT strategies to the patients through the use of culturally relevant metaphors. The following example case vignette illustrates how this might be done:

Mr. Yu is a 55-year-old patient reporting a rapid heartbeat and sweating during the night. He is also a chronic smoker, and says that without smoking, it would be difficult for him to remain calm and comfortable because of the business of his job. Chronic stress and smoking appear to be affecting his health. He attributes his rapid heartbeat to having too much "yang" (i.e., heat) cultivating food in his diet, and not having enough "yin" (i.e., cooling) food. As his physician, you would like to recommend beginning a smoking-cessation program, but you wonder whether his current coping skills for stress are sufficient for the difficult process of quitting smoking. You would like to recommend a MBSR smoking-cessation program, but at the mention of "mindfulness," Mr. Yu tenses and says he is not sure such activities would work for him.

Now the questions are: How to frame a conversation about stress and the potential benefits of MBSR to this patient? How to address his problems in a culturally responsive manner? We attempt to address these issues as follows:

First of all, Mr. Yu acknowledges his physical symptoms of a rapid heartbeat and sweating, but he is not sure if MBSR would work for him. We could start the conversation with the relationship between a rapid heartbeat and sweating and stress, and how MBSR can reduce stress and therefore the symptoms such as a rapid heartbeat and sweating. To support our point, we could share with him the physiology of stress and the scientific evidence of how MBSR can reduces stress (see the section on *Bodily Responses Associated with the practice of Mindfulness and Acceptance* of this article). We could also

share the efficacy of MBSR for people like him with similar symptoms (based on relevant research studies. See the section on *Efficacy of MABTs for CLD Patients* for some examples).

Second, we should answer any questions that Mr. Yu may have about MBSR and clarify any misunderstandings. We could also take the opportunity to highlight that MBSR is based on the premise that every individual has vast inner resources that, through mindfulness meditation practice, can be mobilized to assist in healing.[33] Mindfulness-based stress reduction can be introduced as a new tool or strategy that can be used to address many of the challenges patients are facing in their daily lives.[20] Further, it could include a variety of formats of meditation and/or mindfulness activities, such as practices of breathing meditation, eating meditation, walking meditation, and mindful yoga.[33] Patients can choose a method that works for them as a simple self-care method in their daily lives. Patients can practice mindfulness when cooking, cleaning, relaxing, walking, jogging, and swimming, etc.

Third, as Mr. Wu attributes his rapid heartbeat to having too much "yang" food and not having enough "yin" food, he seems to think in terms of the popular theory of traditional Chinese medicine, in which balancing one's "yin" and "yang" energy is very important for health. We should respect his cultural belief and approach to health. We can recommend MBSR as a method to complement his effort to balance "yin" and "yang" through diet rather than replacing it. We could use the popular Chinese saying "a peaceful mind leads to a healthy body" to illustrate our point. We should also take the social stigma of mental health patients into consideration. In the Chinese culture, getting treatment for mental health problems may result in potential stigma. Thus, framing the symptoms from physiological and dietary perspectives may help Mr. Wu avoid the potential harm of feeling stigmatized. In this cultural context, we could frame MBSR as an 'educational' process of self-care rather than a 'mental health' intervention.

Fourth, we should also take cultural, linguistic, religious, and individual differences into consideration when recommending and communicating about MBSR. Cultural considerations include cultural beliefs, values, and practices relevant to mindfulness meditation. As an example of cultural consideration, we took cultural beliefs and practices into account in Mr. Yu's case. In religious consideration, we examine whether mindfulness is consistent with one's religious beliefs. In Mr. Yu's case, this does not appear to be a problem. In other cases, we may need to explore with the patients whether they can find similarities between their own religious practice (such as prayer) and mindfulness meditation. If they can, they may be able to benefit from MBSR. If they cannot, they may not be ready to participate in MBSR. For these patients, we could explore what cultural and religious practices in their lives may help them reduce stress. Individual differences include age, gender, lifestyle, physical and mental functioning, the kind of illness, and past experiences. For instance, sitting straightly and quietly for an extended time may be difficult for young children and adults who have back pain. These people may need alternative formats or postures. Furthermore, systemic barriers in the patients' lives should also be considered. For instance, if a patient needs to resolve shelter and medical insurance issues before s/he can focus on MBSR, then we have to think beyond our typical roles by taking steps to help address these issues and make appropriate referrals.[33]

Thus, in the case of Mr. Yu and with any patient, being mindful of not only the socio-cultural characteristics of the patient but of the proposed intervention as well can help increase the match between the intervention and the patient's needs. This aforementioned Cultural Match approach[5,6,7] utilizes the discrepancies between the cultural characteristics of interventions and patients as the guiding information for creating cultural adaptations.[38] Because of the intersectional nature of identity[39] and heterogeneity of individuals within specific cultural groups, it is also important not to make generalized assumptions about the endorsement of cultural beliefs within individuals. Thus, cultural adaptions are best designed through collaborative processes with the patient, wherein dialogue about epistemic factors (i.e., how does the patient define the problem? What does s/he believe will lead to improvements?) and salient cultural variables (e.g., self-orientation, gender roles, acculturation level, etc.) are considered.[38] Examples of cultural adaptations for MABTs could include the use of culturally and/or personally meaningful metaphors for guided imagery exercises, the integration of mindfulness practice with current religious practices, as well as values exploration/cultivation in the face of oppressive experiences.[32]

CONCLUSIONS

The growing interest and research in practices grounded in mindfulness and acceptance is resulting in a number of evidence-based mindfulness therapies for a wide variety of stress-related medical issues. The evidence thus far regarding the adaptation of these therapies for CLD populations is promising, and they would be further strengthened though a continual exploration of the cultural meanings implicit in both MABTs and the experience of individuals practicing such approaches. As we communicate with CLD patients about the efficacy of MABTs, we should remember that beliefs about efficacy are also shaped by cultural beliefs. The more we understand the ontological and epistemological basis for these beliefs, the better we can frame the rationale for our medical interventions in a manner acceptable to the patient. This is especially critical in MABTs, because they in large part depend on the willingness of the individual to explore a novel way of relating to stress.

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CONFLICT OF INTEREST

None.

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Review

Genotyping Technologies and Applications in the Era of Precision Medicine

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Over the past decade, genotyping technologies have revolutionized the genomic research field by providing cost-effective genotyping of tens of thousands to millions of genetic markers at population scale. That became the driving force behind genome-wide association studies. The price of a genotyping chip with ~1 million variants is now less than $50. We now see large biobank studies with genotyping data, including the UK Biobank, the China Kadoorie biobank, and the US Million Veteran Program. Second-generation whole-genome sequencing currently costs less than $1,000; and as third-generation sequencing technologies continue to mature, is there still a bright future for genotyping? In this review, we introduce some basic technological points and outline the current status of genotyping technologies; we then discuss the challenges and opportunities for genotyping in the current state and future of precision medicine.
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INTRODUCTION

Large cohorts with rich genetic and phenotypic data are key for the success of the Precision Medicine Initiative. Since whole genome sequencing (WGS) is still expensive, many large studies have taken advantage of cost-effective arrays to assay single-nucleotide polymorphism (SNP). The UK biobank project[1] in United Kingdom, the VA million Veteran program[2] in United States, the China Kadoorie Study[3] in China and United Kingdom, are a few of the largest ones, which already obtained genome-wide SNP data for over 100,000 samples. Genotyping refers to assays specifically designed to target a genomic point, a signal that is polymorphic in the genome. A SNP is a variation in one nucleotide that occurs at a specific position in the genome, where each variation is detectable within a population (e.g. > 1%). For example, at a specific base position in the human genome, the base C may appear in 90% of individuals, but in the other 10% the position is occupied by base A. Thus there is a SNP at this specific base position, and the two possible nucleotide variations (C vs. A) are called alleles for the position. Most SNPs are bi-allelic, but there also are tri-allelic SNPs. Most SNPs are interrogated with one or two probe sets: one derived from the forward strand sequence and/or one derived from the reverse strand sequence. Fan et al. published an excellent review of highly parallel genomic assays,[4] and the Figure 1 from that paper clearly illustrates the key steps in genotyping, which still underlie today's technologies.

Genotyping is commonly contrasted with sequencing, which reads *all* the data in a base pair sequence. As an analogy, genotyping is like reading certain key words in a book, while sequencing is simply reading an entire book. However, new technologies like "genotyping-by-sequencing" are reducing the differences between these two technologies. The cost of sequencing the first whole genome was around $3 billion and concluded in 2003 after 13 years (http://www.genome.gov/11006943). Since then, the cost of sequencing a genome has been decreasing at a speed exceeding Moore's law.[5] The actual cost of sequencing varies depending on whether all or only some aspects of variables such as logistics, sequencing instruments and other large equipment and indirect costs, quality assessment/control, and data interpretation are included. This led some to state that the real cost of genome sequencing is higher than we thought.[6] However, the combination of technological advancements and competition will undoubtedly continue to drive down costs.

The two basic types of arrays used in genomic analysis are *ordered arrays* and *random arrays*. Most Illumina arrays including the Global Screening Assay (GSA) use random arrays, while Affymetrix arrays are manufactured using a photolithographic process, which produces ordered arrays. Ordered arrays means that the arrays manufactured today, next week, or ten years from now for the same array design are

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exactly identical. On the other hand, random arrays are manufactured by sampling a bead pool, which results in random selection of the probe sequences used. That in turn means that each lot of arrays manufactured is slightly different than the other lots, which can cause differences in the final design, which is not possible with bead array technology. With several of the advantages mentioned for the ordered array, a possible disadvantage of ordered array is that it requires specialized equipment, unlike that used to produce Illumina's random arrays.

CURRENT STATUS OF GENOTYPING TECHNOLOGIES

Illumina and Affymetrix have dominated high-throughput genotyping for the past 10+ years. In 2016, Illumina released GSA, which claims to combine a highly optimized, universal genome-wide backbone, hand-curated clinical research variants, and sample tracking content to produce a highly economical array for population-scale genomics and screening. It uses the 24-sample Infinium HTS format, enabling high content flexibility, throughput capacity, and genotyping accuracy. The latest array from Affymetrix is called the Axiom™ Precision Medicine Research Array (PMRA) and claims to provide the most up-to date content, broadest coverage, and highest accuracy for disease-association studies across populations.[7] In general, both GSA and PMRA arrays include the following SNPs: (1). genome-wide imputation grid; (2). global population specific variants; (3). variants from GWAS Catalog and common cancer variants; (4). Rare functional variants from ClinVAR, ExAC consortium; (5). Variants with pharmacogenomic effects including those from PharmGKB databases; (6). HLA region and CNV variants; (7). Fingerprinting variants.

Although basic array mechanisms have not changed dramatically, technologies do evolve over time. Taking the Affymetrix Axiom array as an example, there are quite a few differences between Axiom and the earlier version of Affymetrix 6.0 array, as shown in **Table 1**.

assay results. For and ordered array such as the Affymetrix axiom array, if part of the content on an array needs to be changed, subsequent designs will always be guaranteed to contain the exact retained subset of content from the original

Table 1. comparison of Affymetrix 6.0 array and the latest Axiom array.

	Affymetrix 6.0	**Affymetrix Axiom**	**Comparison**
Format	Cartridge format	Plate format	Axiom arrays are better suited to high sample throughput
Size	~6 million 5-micron features	1.4 million features	Axiom arrays are physically smaller
Amplification	Use restriction enzymes to simplify the genome before amplification	Use whole genome amplification	Axiom arrays can access more of the genome
Specificity	hybridization only (to 25-mer oligo probes)	hybridization and ligation to 30-mers	Axiom arrays has higher specificity
Dyes	Single color	Two dyes	Axiom arrays need much less number of features for each SNP
Copy number analysis	split about evenly between 900k SNP probesets and 900k single-feature copy number probes	fewer probes and a lower dynamic response to copy number variation	Copy number detection does not work as well on Axiom
SNP contents	Much earlier, more limited design	Reflects current knowledge on human genome	The axiom arrays include more markers to reflect global diversity, and more markers with clinical relevance

CHALLENGES OF SNP GENOTYPING TECHNOLOGIES

A few challenges remain to be resolved by even the most up-to-date genotyping technologies. They include but are not limited to direct assays of haplotype, copy number variations (CNVs), and human leukocyte antigen (HLA) region.

Directly Assaying Haplotype

Haplotype information is very important, which is usually not directly captures by genotyping technologies. Take the well-known *APOE* gene for example. The ε4 haplotype is defined by two variants: rs429358-C (build 37 position 45,411,941), rs7412-C (build 37 position 45,412,079). It has been implicated in a variety of diseases, including atherosclerosis,[8] AD,[9] impaired cognitive function,[10] reduced hippocampal volume,[11] HIV,[12] faster disease progression in multiple sclerosis,[13] unfavourable outcome after traumatic brain injury,[14] sleep apnea,[15] accelerated telomere shortening.[16] The current Axiom arrays have probes that directly assay these two key ApoE variants (rs7412 and rs429358), and it is actually claimed to be the only arrays on the market that can reliably assay these two variants. This is primarily due to Axiom array's capacity to use both hybridization and ligation instead of hybridization alone technology to tackle the genome surrounding these two SNPs with high GC content in the flanking regions. But still, the Axiom assays can only assay these two variants separately, not able to directly assay the haplotype built from these two variants. Instead, statistical phasing software is used to determine the haplotype of these two SNP.[17]

For haplotypes composed of SNPs that are very close to each other (e.g., within 15-20 bp), they can actually be directly detected using current genotyping technologies. If the two

APOE SNPs were only 10 bp apart (rather than 138 bp, as is the case), we could design four probes, one matching each haplotype (or two probes, taking advantage of the two-color system). This would essentially be multiallelic genotyping of a four-allele variant. However, variants more than about 15 bp apart cannot successfully be combined this way, since with hybridization the 30-mer Axiom probes would be increasingly less specific with distance past that point. Recently, fluorescence in situ hybridization (FISH) is used as a powerful single-cell technique for directly assaying haplotypes. Beliveau et al. introduced a robust and reliable system that harnesses SNPs to visually distinguish between the maternal and paternal homologous chromosomes in both mammalian and insect systems.[18] The method makes use of Oligopaints, which are highly efficient, renewable, strand-specific FISH probes derived from complex single-stranded DNA (ssDNA) libraries in which each oligo carries a short stretch of homology to the genome. An open-jaw molecular inversion probe [19,20] could also be a promising approach for directly assaying haplotypes. Assuming the homology arms could be designed and a ~120bp (i.e., 138bp minus 15-20bp) gap-fill would work, we could then design four probes, one for each haplotype.

Directly Assaying Copy Number Variation (CNV)

Currently, CNV detection does not work very well with sequencing, but it does work with SNP arrays. This is because it requires impractically high depth of sequencing to obtain accurate CNV signal, which genotyping array captures CNV signal naturally. A double deletion is easy to distinguish from two copies, but the ability to call one or three copies requires a good dynamic range of response in signal or reads. The problem becomes even more complex for mosaic samples, e.g., a tumor sample with only a fraction of the cells having an aberration. A microarray has thousands (or tens of thousands) of probes in each feature, which inherently provides a practically continuous response and the possibility of high dynamic range and good signal-to-noise. Achieving the same accuracy with sequencing requires many more reads than are necessary for genotyping, therefore making genome-wide CNV detection very expensive.

Directly Assaying the Human Leukocyte Antigen (HLA) Region

The human leukocyte antigen (HLA) complex is the human version of the major histocompatibility complex (MHC) region in chromosome 6, which includes genes responsible for immune function. Variations in these genes affect immune response, including those responsible for transplant rejection as well as disease susceptibility. The naming of HLA variants is quite complex. All alleles start with "HLA", and the next portion (HLA-**A** or HLA-**B**) identifies the gene of which the allele is a modification. The next two numbers (HLA-A***02**) signify what antigen type that particular allele is, typically the serological antigen present. In other words, HLAs with the same antigen type (e.g., HLA-A*02:101 and HLA-A*02:102) will not react with each other in serological tests. The next set of digits (HLA-A*02**:101**) indicates what protein the allele codes for; these are numbered sequentially based on the order in which they were discovered. The third set of numbers (HLA-A*02:101**:01**) indicates an allele variant that has a different DNA sequence but produces the same protein as the normal gene. The final set of numbers (HLA-A*02:101:01**:01**) designates whether there is a single or multiple nucleotide polymorphism in a non-coding region of the gene. The final aspect of HLA naming is one of six letters (for example, HLA-A*02:101:01:01**L**). The letter L in this example means lower-than-normal cell surface expression.

The highly polymorphic nature of the HLA region and the prevalence of pseudogenes create challenges for traditional genotyping methods. Combining direct genotyping with advanced imputation methods over the extended MHC region allows accurate HLA typing from SNP genotype data. For HLA-specific markers, Affymetrix provides a tool that uses directly assayed genotypes from the Axiom array to impute and generate two- and sometimes four-digit HLA resolution. In contrast, Illumina claims that its TruSight HLA Sequencing Panel delivers unprecedented accuracy, efficiency, and certainty in HLA typing, all in one assay. It is also worth mentioning SNP2HLA, developed by the Broad Institute (http://software.broadinstitute.org/mpg/snp2hla/). It imputes not only the classical HLA alleles but also the amino acid sequences of those classical alleles, so that individual amino acid sites can be directly tested for association. This allows for facile amino acid-focused downstream analysis.[21]

THE FUTURE OF GENOTYPING TECHNOLOGIES

Before we discuss the cost and effectiveness of genotyping vs. sequencing, it is important to keep in mind that only sequencing can detect novel variants. However, sequencing the whole genome for $100 is not yet a reality and probably will not be accessible to anyone outside of the largest sequencing labs for at least a few years. Also, accurate detection of rare content requires deep sequencing, which generates enormous amounts of data and requires weeks to months of analysis to generate usable results. We think that genotyping technologies will remain the platform of choice for many years, for at least the following reasons: 1) they are very affordable; 2) they take relatively little time to quality-control, filter, and generate genotypes (~1.5 hours for a plate of 96 samples); 3) they can be easily customized to meet virtually any need, and 4) they can generate data on hundreds of samples per week. The possible uses for genotyping in the era of precision medicine includes but are not limited to comprehensive assays of blood types, diseases in newborns and variants recommended by the American College of Medical Genetics (ACMG), and fast genotyping for detecting pathogens and in point-of-care settings.

Comprehensively Assay Blood Types, Something That Everybody Cares

Red blood cells (RBCs) carrying a particular antigen may elicit an immune response if introduced into the blood circulation of a patient who lacks this antigen. It is the antibody produced during the immune response that is problematic and leads to donor/patient transfusion incompatibility, maternal-fetal incompatibility, and autoimmune hemolytic anemia. This

immune response can be immediate or delayed and may in some cases be lethal. Knowing one's blood type is important for both scientific and medical purpose. People with non-O blood types have an increased mortality particularly due to cardiovascular diseases. This is partially due to the effect of blood group alleles on blood biochemistry including von Willebrand factor and factor VIII levels.[22] As genotyping becomes cost-effective and more easily automated and multiplexed than phenotyping, there is a desire to derive human blood type from genetic data. Also, blood typing through genetics does not really need blood. As of today, there are 346 serologically distinct red blood cell (RBC) blood group antigen phenotypes recognized by the International Society of Blood Transfusion (ISBT),[23] defined by over 1,100 alleles across 45 genes (http://www.isbtweb.org/). There are 33 serologically distinct human PLT antigen (HPA) phenotypes (http://www.ebi.ac.uk/ipd/hpa/), defined by 33 alleles within six genes. Centralized efforts have been put to catalog these genetic variants, including the ISBT website, the BGMUT website,[24] the *RHD* RhesusBase (http://www.rhesusbase.info/), and the Immuno Polymorphism Database-HPA website.[25]

However, in reality, even for the well studied ABO blood types, genetic data is rarely used to determine its type. One obstacle is that ABO polymorphic sites associated with antigen expression are documented according to nucleotide positions in cDNA, not genomic coordinates. The other obstacle is the complex link between the genetic variations and the resulting blood types. Take ABO blood type as an example. It is one of the RBC carbohydrate antigens (together with $Le^{a/b}$, P1, P^k) synthesized by enzymes, and it requires gene sequencing to properly predict the enzymatic and sugar specificity across several genes. The ABO gene has seven coding exons, with the majority of the coding sequence lying in exon 6 and 7. Four common missense variants in exon 7 that differentiate between the A and B haplotype result in amino acid substitutions in the active/binding site of the ABO glycosyltransferase: rs7853989 (p. R176G), rs8176743 (p. G235S), rs8176746 (p. L266M), and rs8176747 (p. G286A).[26,27] An exon-6 deletion (rs8176719) leads to the classic O genotype and phenotype, while another common deletion located at the end terminus of exon 7 (rs56392308) results in the A2 subtype.[28] For decades, the method of reference for testing blood group antigens was the hemagglutination technique. This is a simple and well-established technique usable for all major blood groups, with specificity, sensitivity, and security appropriate for the clinical diagnostic environment. However, this gold-standard method has certain limitations when it comes to the determination of minor or rare blood group antigens critical to determine a perfect match between patient and donor, including immunologic reagent availability and specificity.[29] More comprehensive evaluation of the performance of genetically predicated blood types would contribute to transfusion medicine and therefore precision medicine. While realizing the great potential of using genetic data to predict blood group, we don't recommend it to replace the conventional serological methods yet, because the clinical significance of missing one inactivating mutation for the ABO blood type would pose an unacceptable risk for transfusion.

Comprehensively Assay Newborn Diseases Pathogenic Variants

Newborn screening tests provide an early opportunity to detect certain disorders before symptoms appear. At about 48 hours after birth, or just before a baby is discharged from the hospital, a small blood sample is taken and tested for a variety of conditions/disorders. At the state government level, usually an advisory board made up of doctors, nurses, scientists, ethicists, and parents advises which disorders to include. For a disorder to be included in the list, the following must be true: 1) the disorder is treatable, 2) there is a good test, and 3) early medical intervention would benefit the infant. For example, 32 disorders are included in routine screening mandated by the Massachusetts Department of Public Health (http://nensp.umassmed.edu/screening-programs/massachusetts/routine-disorders).

In 2013, the American College of Medical Genetics and Genomics (ACMG) released a guideline [30] that recommends clinical diagnostic laboratories performing exome or genome sequencing to report known pathogenic or expected pathogenic variants within 56 genes even when unrelated to the primary medical reason for testing. Subsequently, the ACMG revised the terminology from "incidental findings" to "secondary findings" because these genes are intentionally being analyzed, as opposed to genetic variants found incidentally or accidentally. The shift in terminology also maintained consistency with a recommendation by the Presidential Commission on Bioethical Issues.[31] An additional modification to the original policy included offering an option for individuals undergoing clinical genomic sequencing to opt out of receiving secondary findings. The updated list includes 59 medically actionable genes recommended for returning the genetic results to patients who participated clinical genomic sequencing.[32]

Fast Genotyping for Detecting Pathogens and Point-Of-Care Settings

In April 2017, CRISPR pioneering Dr. Feng Zhang and colleagues at the Broad Institute reported a CRISPR-based diagnostic tool that can detect pathogens, identify cancerous mutations, and genotype human DNA.[33] The tool is called SHERLOCK, for Specific High Sensitivity Enzymatic Reporter UnLOCKing. also includes a reporter RNA strand that fluoresces when cleaved. When Cas13a detects the targeted RNA sequence, its unbiased RNAse activity will slice the reporter sequence, releasing a detectable fluorescent signal. Cas13a). This new tool incorporates isothermal RNA amplification that was previously used to create a paper-based Zika test, and it is now capable of detecting single RNA and DNA molecules at attomolar concentrations. The other benefits include quick turn-around time (less than 1 hour), portable, and low-cost (less than $1 a sample). All these features are key to build a genotyping tool that can reach far beyond research labs and make true difference for both public health and precision medicine.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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Review

Can Transarterial Chemoembolization of Hepatocellular Carcinoma Result in Transformation to Combined Hepatocellular-Cholangiocarcinoma with Stem Cell Features? A Case Study

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Transarterial chemoembolization (TACE) is currently one of the favored treatment modalities for non-curative hepatocellular carcinoma (HCC) and can be used to shrink tumor size in order to make a patient eligible for transplantation. Furthermore, with the advent of effective antiviral drugs for hepatitis B virus (HBV), concomitant antiviral therapy with local tumor ablation including TACE for HBV-associated HCC has been successful for long term survival. Over the last decade, however, concern has been raised about a phenomenon whereby a subset of TACE-treated HCCs becomes more aggressive after TACE treatment. One current hypothesis is that TACE eliminates only the hepatocellular cells and that hepatic progenitor cells that have the potential for developing to cholangiocarcinoma are then selected for and induced to proliferate post-TACE with possible dual differentiation along hepatocellular and biliary lines. We present a case of a patient with HCC who underwent TACE and subsequently experienced tumor regrowth as biopsy-proven combined hepatocellular-cholangiocarcinoma. Furthermore, we provide immunohistochemical evidence of hepatic progenitor cells in the post-TACE tumor biopsy, possibly accounting for its aggressive course.
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Key Words: *transarterial chemoembolization, hepatocellular carcinoma, stem cell*

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the third cause of cancer-related mortality globally.[1] While liver transplantation is the therapy of choice, the shortage of organ supply has been one of the major difficulties for patients in need. Since effective drugs for hepatitis B virus (HBV) have become available, concomitant anti-HBV therapy with local tumor ablation or resection has demonstrated significantly improved survival of these patients, as summarized by Yuan et al.[2] In fact, our institution (Thomas Jefferson University Hospital) has reported the longest survival of HBV-HCC patients with the above treatment modality.[3] Transarterial chemoembolization (TACE) is frequently used for local tumor ablation. It is also the favored treatment for non-curative HCC and can be used to shrink tumor size in order to make a patient eligible for transplantation. The procedure achieves cytoreduction or eradication through both ischemic and chemotherapeutic means. TACE has shown objective response rates in 16-60% of patients, with complete response achieved in less than 2%.[3] Over the last decade, however, concern has been raised about a phenomenon whereby a subset of TACE-treated HCCs becomes more aggressive after TACE treatment. One current hypothesis is that TACE eliminates only the hepatocellular cancer cells and that hepatic progenitor cells that have the potential for developing into cholangiocarcinoma are then selected for and induced to proliferate post-TACE with possible dual differentiation along hepatocellular and biliary lines. We present a case of a patient with HBV-associated HCC that underwent TACE and then experienced tumor regrowth as combined hepatocellular-cholangiocarcinoma (HCC-CC). Furthermore, we provide immunohistochemical evidence of hepatic progenitor cells (HPCs) in the post-TACE tumor biopsy, possibly accounting for its aggressive course.

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CASE REPORT

A 26 year-old Asian male was noted to be HBsAg (+) at age 26 during circumcision in 11/2005. Family history revealed his mother negative for HBsAg and anti-HBs but positive for anti-HBc. His maternal grandmother was HBsAg (+). Also his paternal great uncle had hepatitis B with cirrhosis. He was likely to have been infected during early childhood. He was HBeAg (+) with HBV DNA $2.8x10^5$copies/ml. He was started on adefovir by his hepatologist.

In 8/2006, MRI showed nodular liver with splenomegaly and paraesophageal varices. There was no tumor seen imaging. Serum albumin was 3.9 gm/dl, ALT 66 U/L, platelets 94,000/mm^3, HBeAg (+) and HBV DNA was $1.4x10^3$ copies/ml. Lamivudine was added to adefovir.

In 3/2007, MRI showed a 1.2cm HCC in the right posterior lobe (segment 7). AFP was 2.9 ng/ml. Options on various therapies including local ablation, liver transplantation, and resection were discussed and the patient opted for local ablation. He said he would rather take multiple tumor ablations than undergo transplantation. He received successful percutaneous ethanol injection. He remained tumor free for 3 years until 7/2010 when MRI showed a new 1.3 cm enhancing tumor mass in the right lobe (segment 6). AFP was 442 ng/ml with 83.6% AFP-L3 indicative of HCC.

Following discussion with the patient and his family, the patient opted for TACE. However, 10 months later (7/2011), MRI showed a 3.1 cm recurrent tumor at the treated site. On his insistence for a local procedure rather than transplantation, he underwent TACE followed by laparoscopic microwave ablation in 9/2011. AFP was 517 ng/ml with AFP- L3 84.8 %. He was started on sorafenib. Sorafenib was poorly tolerated, and in 7/2012 after clinical progression he was enrolled in a Phase 2 clinical trial testing a new agent for metastatic HCC. AFP was 8344 ng/ml. At the first clinical evaluation he showed significant progression in the liver with increase in AFP to 29, 373 ng/ml and liver biopsy was obtained in 10/2012. The result was combined HCC-CC.

Further immunochemical studies were carried out as described below.

METHODS

For the purpose of this study, we confirmed the dual differentiation of the tumor by reviewing the hematoxylin and eosin (H&E) stained slides of the post-TACE liver biopsy. In addition, evaluation for HPCs was done by immunohistochemical staining for EpCAM, NCAM, CK19, CK7, and hepatocyte specific antigen (HSA) on formalin-fixed, paraffin-embedded tissue (**Table 1**) using the VentanaUltraview detection kit (Ventana Medical Systems, Tucson, AZ) with the BenchMark Ultra IHC staining module. Five micron sections were cut using a microtome. The slides were deparaffinized in a dry oven at 72°C for 20 minutes. They were then placed into a tris-based buffer with basic pH called ULTRA cell conditioner #1 (Ventana, catalog #950-224). The slides were then brought to 36-37°C and incubated for four minutes. Specific antibodies were then added as follows: anti EpCAM (clone BerEP4), anti-CD56 (clone 23C3mAb), anti-CK19 (clone A53-B/A2.26), anti-CK7 (clone SP52), and anti-HSA (clone OCH1E5). For staining amplification, an additional step for anti-CD56 was required where mouse antibody was added and allowed to incubate at 36°C for an additional 12 minutes. Excessive antibody was then washed off using ultraWash solution (Ventana Medical Systems). The slides were then counterstained with hematoxylin and incubated for 8 minutes. The stained slides were examined under a microscope for nuclear, cytoplasmic, and/ or membranous staining of the morphologically glandular and hepatocellular components to determine positivity or negativity. The criteria for positivity were the percentage of cells positive within the lesion: < 5% = negative, 5-24% = 1+, 25- 49% = 2+, and 50% or more = 3+ positivity. Intensity of staining was not taken into account for the purpose of this study.

RESULTS

The tumor displayed both an infiltrative glandular component in the form of tubules with lumens and moderate nuclear atypia intermixed with a well-differentiated hepatocellular component in the form of thickened plates of hepatocytes in a trabecular pattern, increased nuclear size, and prominent nucleoli (**Figure 1, A-B**). As expected, HSA antibody showed expression in the HCC component with 1+ cytoplasmic staining while the glandular component showed no positive staining (**Figure 1, C-D**). EpCAM expression was diffusely 3+ positive in the cytoplasm of the HCC component and 1+ positive in the glandular component (**Figure 1, E-F**). NCAM showed 3+ positivity in the cell membranes and cytoplasm of glandular components while the HCC component was completely negative (**Figure 2, A-B**). CK19 showed diffuse cytoplasmic staining in not only the malignant glandular component but also in the well differentiated HCC component (3+ positivity in both areas, **Figure 2, C-D**). CK7 was expressed within the cytoplasm of the tumor cells in the HCC component with 2+ positivity and in the glandular component with 3+ positivity (**Figure 2, E-F**). In summary, the post-TACE tumor biopsy showed morphology of HCC-CC and revealed expression of stem cell markers EpCam, NCAM, CK19, and CK7 by immunohistochemistry (**Table 2**).

DISCUSSION

We present a patient with HBV infection and radiologically-confirmed HCC who underwent TACE, whose post-procedure course was notable for a tumor with rapid growth and increased aggressiveness that eventually resulted in the patient's death. Biopsy of his tumor post-TACE revealed combined HCC-CC with stem cell features, including expression of immunohistochemical markers associated with hepatic progenitor cells (HPCs), specifically EpCam, NCAM, CK19, and CK7.

Combined HCC-CC is a relatively rare tumor, comprising less than 1% of all liver carcinomas.[4] As defined by the World Health Organization, combined HCC-CC is classically defined as having areas of typical HCC and areas of typical CC

intimately mixed.[4] If tumor cells that have a morphology or immunophenotype of stem cells/HPCs predominate, the term "combined HCC-CC with stem-cell features" is recommended.[4] There is growing evidence that the TACE procedure may cause transformation of typical HCC to combined HCC-CC with stem cell features. No clear consensus exists on which markers to use for hepatic stem cells/HPCs. CK7 is considered a marker of intermediate hepatocytes (a committed hepatocyte precursor) and CK19 a marker of HPCs.[5-7] Nishihara et al[8] showed that HCC treated with TACE had significantly increased CK19 expression compared to untreated HCC. This was expanded upon by Zen et al[9] who showed significantly increased expression by immunohistochemistry and RT-PCR for CD133, CK19, EpCAM, and NCAM in TACE-treated tumors versus untreated tumors, as well as increased HCC-CC in TACE-treated tumors. Zeng et al[10] showed similar results, with TACE-treated tumors showing significantly increased EpCAM and CD133 expression compared to untreated tumors. Importantly, the above studies excluded any cases that showed either radiologic or biopsy evidence of combined HCC-CC prior to the TACE procedure. Therefore, our case supports the growing evidence that a subset of TACE-treated tumors may transform to combined HCC-CC and the increased expression of markers associated with HPCs implicates stem cells in this process.

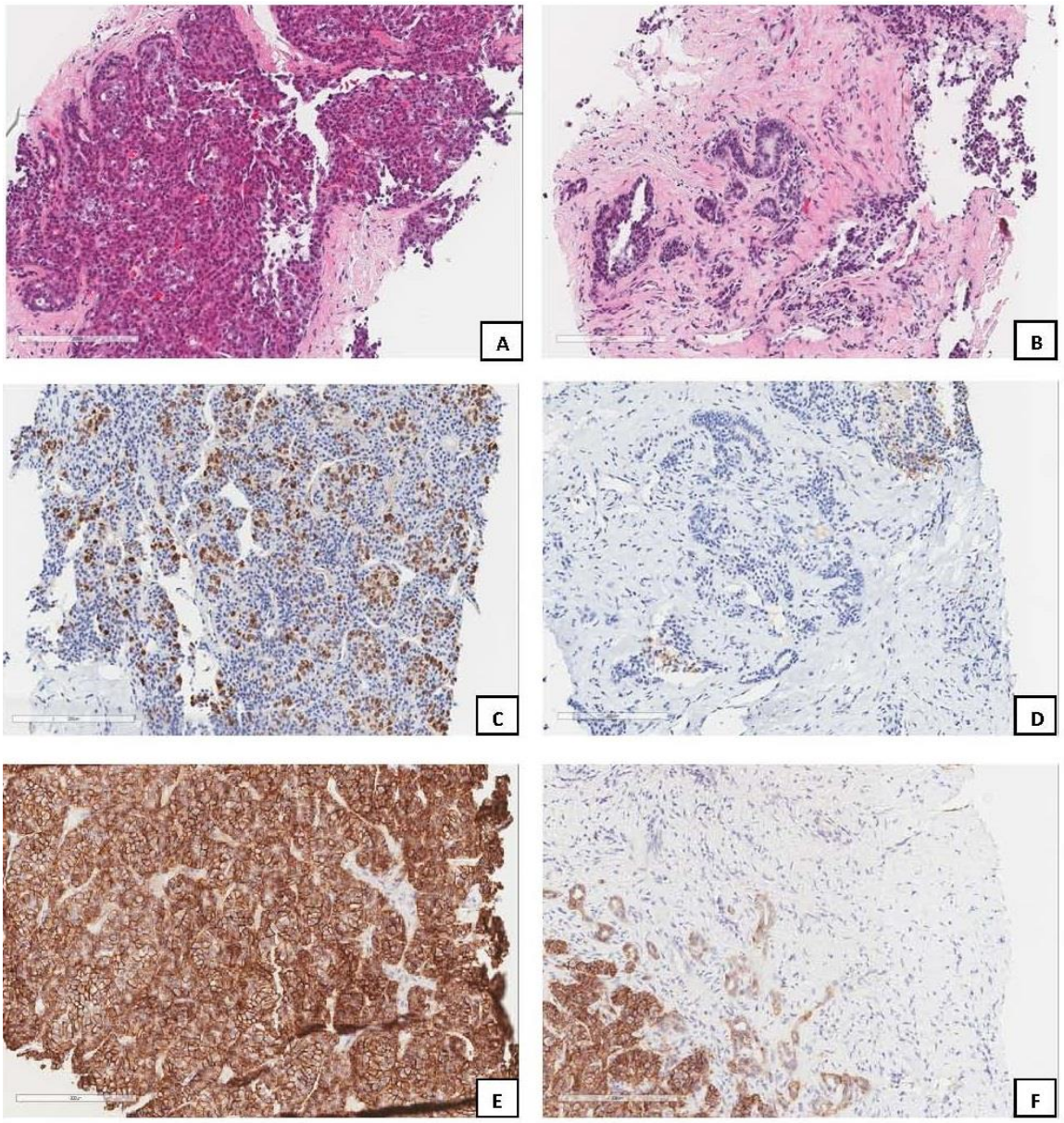

Figure 1. A) Well differentiated hepatocellular carcinoma component, H&E 200x **B)** Cholangiocarcinoma component, H&E 180x **C)** and **D)** HSA immunohistochemical (IHC) stain with 1+ positivity in HCC (C) and negative HSA IHC in cholangiocarcinoma component (D). **E)** and **F)** EpCAM IHC stain with 3+ positivity in HCC component (E) and 1+ positivity in cholangiocarcinoma component (F).

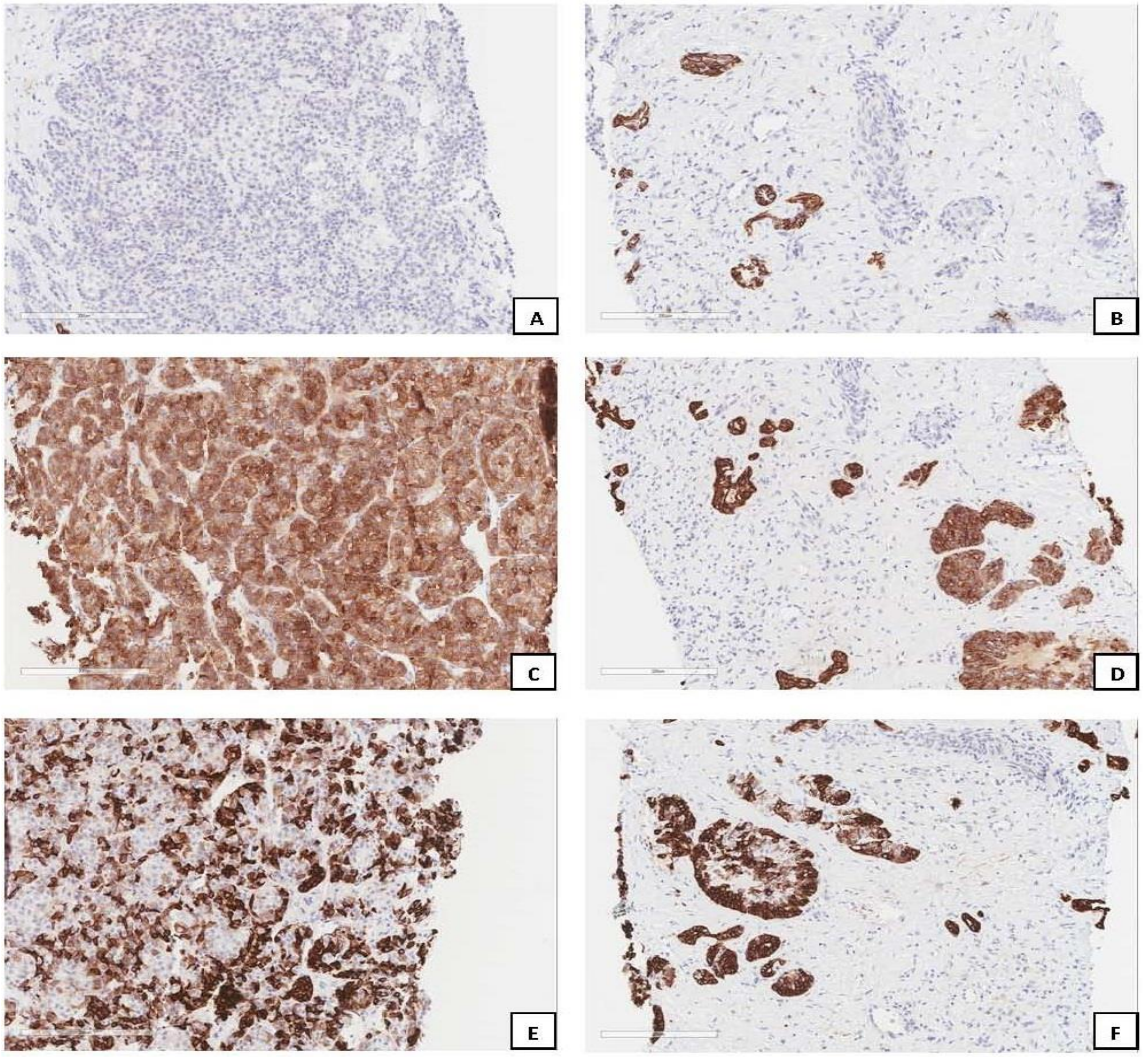

Figure 2. A) Negative NCAM IHC stain in the HCC component, 200x **B)** NCAM IHC stain with 3+ positivity in the cholangiocarcinoma component, 200x **C)** and **D)** CK19 immunohistochemical (IHC) stain with 3+ positivity in HCC **(C)** and cholangiocarcinoma component (D). **E)** and **F)** CK7 IHC stain with 2+ positivity in HCC component (E) and 3+ positivity in cholangiocarcinoma component (F).

The true significance of increased HPC marker expression in TACE-treated tumors lies in the more aggressive tumor biology. Several studies have shown that TACE-treated HCC tumors expressing CK7, CK19, CD133, and/or EpCAM - markers associated with HPCs - show significantly increased recurrence rates after transplant compared to untreated tumors.[9-13] Why a therapy that has been shown to shrink and rarely eradicate HCC tumors should result in increased aggressiveness in a subset of patients is not currently known. Several studies have shown that HCCs treated with TACE have residual viable tumor most of the time.[14-17] Moreover, there is evidence of increased proliferation of both intratumoral endothelial cells and tumor cells in TACE-treated tumors compared to untreated tumors, as measured by Ki-67 immunohistochemistry.[18]

A current unifying theory to explain these findings suggests the residual viable tumor cells after TACE contain a population of chemotherapy- and/or ischemia-resistant HPCs that are induced to undergo proliferation after TACE.[9,10,19]

Table 1. Immunohistochemical stains with staining patterns and cellular localization.

Immunohistochemical stain	Cellular localization	Staining pattern
Cytokeratin 7 (CK7)	cytoplasmic	Positive in hepatic stem cells, benign biliary ducts and cholangiocarcinoma; positive in HCC in only 20% of the cases
Cytokeratin 19 (CK19)	cytoplasmic	Positive in hepatic stem cells, benign biliary ducts and cholangiocarcinoma; usually negative staining in HCC
NCAM (CD56)	membranous /cytoplasmic	Positive staining in progenitor cells committed to biliary lineage and reactive biliary epithelium
EPCAM (BerEP4)	membranous	Positive in hepatic stem cells; negative in mature hepatocytes
HSA (HepPar1)	cytoplasmic	Positive in benign and malignant hepatocytic tumors-hepatoblastoma, hepatic adenoma, HCC; negative staining in cholangiocarcinoma

Table 2. Stem cell marker expression in combined HCC-cholangiocarcinoma.

Antibody	Location	HCC component	Cholangiocarcinoma component
CK7	cytoplasmic	2+	3+
CK19	cytoplasmic	3+	3+
NCAM (CD56) /	membranous /cytoplasmic	Negative	3+
EpCAM	Membranous	3+	1+
HSA	cytoplasmic	1+	Negative

*The expression was considered positive or negative based on the percentage of cells that expressed the antigen in the lesion: < 5% = negative expression, 5-24% = 1+, 25-49% = 2+, ≥ 50%= 3+.

These HPCs are capable of bipotential differentiation into hepatocytic and cholangiocytic/biliary phenotypes, thus accounting for the combined tumor morphology. Therefore, a combination of treatment resistance and increased proliferation may account for the development of combined tumors with stem cell features that arise after TACE therapy with poor prognosis.

Our case highlights the potential for TACE therapy to result in an unintended consequence of increased tumor aggressiveness. The exact incidence of this occurrence is unknown but is relatively rare. Our service performs approximately 200 TACE procedures for HCC each year and less than five HCC-CC tumors have been identified in the last five years. Unfortunately, a pre-TACE biopsy of this patient's tumor was not obtained to allow comparison with the post-TACE sample; thus, we are not able to definitively show a histologic and immunophenotypic transformation. This is commonly the case in clinical practice, as HCC has diagnostic imaging characteristics that obviate the need for a tissue diagnosis.[3] In the current case, the diagnosis of HCC was supported by his AFP level of 517 ng/ml with AFP- L3 84.8 %.[20,21] Based on this observation, the possibility of transformation of HCC to CC or combined HCC-CC should be considered if TACE treated HCC becomes aggressive and poorly responsive to treatment. Future research is needed to address three questions: Is performing HPC marker immunohistochemistry on a pre-TACE biopsy useful to predict recurrence after treatment? Which patients might benefit from post-TACE tumor biopsies with HPC markers performed to assess for tumor transformation? Given the small but possible risk of tract seeding from biopsy, is there a potential role for liquid biopsy to determine tumor aggressiveness?

CONFLICT OF INTEREST
None.

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