ISSN 2186-3644 Online ISSN 2186-361X

IRDR

Intractable & Rare Diseases Research

Volume 7, Number 2
May, 2018

IRCA BSSA

IACMHR

www.irdrjournal.com

IRDR
Intractable & Rare Diseases Research

ISSN: 2186-3644
Online ISSN: 2186-361X
CODEN: IRDRA3
Issues/Year: 4
Language: English
Publisher: IACMHR Co., Ltd.

Intractable & Rare Diseases Research is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group and is published quarterly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA, Shandong Academy of Medical Sciences, and Shandong Rare Disease Association.

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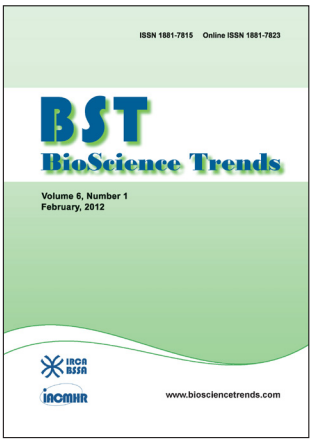
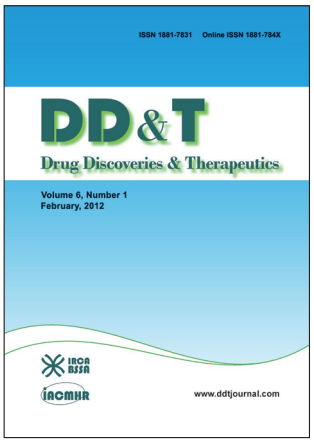
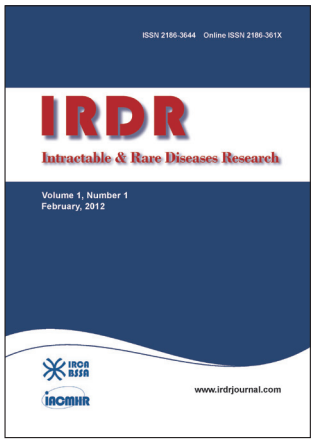


ISSN: 1881-7815
Online ISSN: 1881-7823
CODEN: BTIRCZ
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.
www.biosciencetrends.com



ISSN: 1881-7831
Online ISSN: 1881-784X
CODEN: DDTRBX
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.
www.ddtjournal.com



ISSN: 2186-3644
Online ISSN: 2186-361X
CODEN: IRDRA3
Issues/Year: 4
Language: English
Publisher: IACMHR Co., Ltd.
www.irdrjournal.com

Intractable & Rare Diseases Research

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(*This journal was partially supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science.*)

Editorial

DOI: 10.5582/irdr.2018.01048

Modeling the rare diseases process in dish

Wei Tang*

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Summary **For rare diseases, how to mimic the pathological progression is of importance for better understanding the molecular mechanism and identifying potential targets. Induced pluripotent stem cells (iPSCs) technology provides an ideal model to in vitro obtain the diseases-associated cells. In this issue of *Intractable & Rare Diseases Research*, two birth detect diseases iPS models on phenylketonuria and trisomy 18 were established, and have demonstrated great potential in the research on these rare diseases.**

Keywords: Rare diseases, cell model, induced pluripotent stem cells, phenylketonuria, trisomy 18

The opportunity of obtaining pathological samples from a rare disease patient is extremely rare. Therefore, most of the current researches focus on the gene knockout mice models, however, what extent of these models can reflect the aspects of rare diseases is always difficult to be evaluated accurately.

During the past decade, the development of induced pluripotent stem cells (iPSCs) technology provides an overall new strategy to reproduce the pathological progress of diseases in a dish (*1*). Human iPSCs can derived from many obtained somatic cells from patients such as surgically resected skin fibroblasts, blood mononuclear cells, urine cells or body liquid cells, et al. The iPSCs from rare disease patients have the capacity for self-renewal and pluripotency, theoretically are well suited to modeling the disease process of donors. Using the iPSCs-based models, a series of new molecular mechanisms of rare diseases has been identified (*2*). These in dish rare disease models are superior to other strategies in the following aspects: first, the most disease-associated cell types can be obtained step by step from iPSCs; secondly, the cell models carry all the genetic and even part of epigenetic information of the patients; thirdly, donor sources of iPSCs are safely, affordably, and always frequently obtained.

In this current issue, two original articles described their work on the establishment of rare diseases models. Xing *et al*. (*3*) induced a typical human trisomy 18 induced pluripotent stem cell line from amniotic fluid cells. Different previous unsuccessful effort, this model retained stable trisomy 18 trait after 1ong generation of passages. In another paper, Qi *et al.* (*4*) generated a urine-derived iPSCs from a patient with Phenylketonuria (PKU). PKU-iPSCs have been established previously, this is the first time PKU-iPSCs were induced from the urine cells of PKU patients.

The use of patient-specific iPSCs for modeling rare diseases has demonstrated its potential to provide a better understanding of the exact pathological mechanisms of rare diseases, and is becoming more routine and more scalable. *Intractable & Rare Diseases Research* will continue to focus on more efforts to introduce most updated knowledge and original research in this field in the future.

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(Received May 25, 2018; Accepted May 28, 2018)

Mini-Review

DOI: 10.5582/irdr.2018.01026

Methylmalonic acidemia: Current status and research priorities

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Summary **Methylmalonic acidemia (MMA) is a lethal, severe heterogeneous disorder of methylmalonate and cobalamin (cbl; vitamin B12) metabolism with poor prognosis. Two main forms of the disease have been identified, isolated methylmalonic acidurias and combined methylmalonic aciduria and homocystinuria, which is respectively caused by different gene mutations. Here, we review the improvement of pathogenesis, diagnosis and treatment in MMA. Importantly, the reported epidemiological data of MMA patients in China and the hot mutation sites in Chinese patients are listed, which will aid in improving healthcare of Chinese patients in the future. c.729_730insTT was the most common mutation in Chinese isolated MMA patients, while c.609G>A and c.658_660delAAG were in Chinese cblC type patients according to unrelated studies. The estimated newborn screening incidence was reported to be 1:26,000, 1:3,920, 1:11,160, 1:6,032 respectively in Beijing and Shanghai, Shandong province, Taian district, and Henan province of China. Alternatively, when patients with suspected inherited metabolic diseases were used as the screened sample, the relatively high incidence 0.3% and 1.32% were respectively obtained in southern China and throughout all the provinces of mainland China and Macao with the exception of five provinces (Hainan, Neimenggu, Tibet, Ningxia, and Hong Kong).**

Keywords: Methylmalonic acidemia, hot mutation sites, diagnosis, treatment, epidemiological data, China

1. Introduction

Methylmalonic acidemia (MMA) is a lethal, severe and multi-systems injured disease of abnormal metabolism. It was first reported in 1967 (*1*). According to phenotype, two main forms of the disease have been identified, including isolated methylmalonic acidurias and combined methylmalonic aciduria and homocystinuria. Isolated methylmalonic acidurias is due to defects of methylmalonyl-CoA mutase or the synthesis of the MUT coenzyme adenosylcobalamin (AdoCbl), while combined methylmalonic aciduria and homocystinuria is characterized by elevated plasma homocysteine and decreased levels of the coenzymes adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl). Additionally, some benign MMA subtypes have been described.

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MMA has a wide clinical spectrum, ranging from a benign condition to fetal neonatal disease. Onset of the manifestations of MMA ranges from the neonatal period to adulthood. Affected children usually exhibit anorexia, failure to thrive, hypotonia, developmental delay, progressive renal failure, functional immune impairment, optic nerve atrophy, and hematologic abnormalities. 4 children with combined MMA and homocysteinemia were recently reported to present predominantly with late-onset diffuse lung diseases (DLD) (*2*). Atypical and "benign"/adult MMA are associated with increased, albeit mild, urinary excretion of methylmalonate; however, it is uncertain whether individuals with these conditions will develop symptoms.

Patients with MMA experience significant morbidity and mortality, and the prognosis for long-term survival is poor. The mortality of mut MMA was 60-88% in the first reports in the 1980s and 1990s (*3*) and has improved somewhat to ~40% by the first decade in the 2000s (*4*). A prospective cohort study was conducted in 45 Chinese pediatric patients diagnosed

with methylmalonic acidemia with homocystinuria between 2006 and 2012, which show that elevated urea and urinary methylmalonic acid are predictors of adverse outcomes for the patients (*5*).

2. Etiology and pathogenesis

Methylmalonic aciduria is a genetically heterogeneous disorder of methylmalonate and cobalamin (cbl; vitamin B12) metabolism.

Isolated methylmalonic acidurias have also been classified by complementation groups: MMA 'mut' , caused by mutation in the *MUT* gene on chromosome 6p21; MMA cblA, caused by mutation in the *MMAA* gene on 4q31; MMA cblB, caused by mutation in the *MMAB* gene on 12q24; and Tcblr type, caused by mutation in the *CD320* gene on chromosome 19p13.2. MMA 'mut' involves two subtypes: Mut0 mutations result in no detectable MCM activity; Mut- mutations result in low residual enzyme activity. MCM, an adenosylcobalamin-dependent enzyme, have a function in isomerization of L-methylmalonyl-CoA to succinyl-CoA, for entry into the tricarboxylic acid cycle for energy production. All mutations of *MMAA* strongly decreased functional association with MUT and interfered with gating the transfer of AdoCbl from MMAB to MUT, which is a disease-causing mechanism of cblA type MMA (*6*). The *MMAB* gene encodes the mitochondrial enzyme ATP: cobalamin adenosyl transferase (ATR), which catalyzes transfer of an adenosyl group from ATP to cobalamin (I) to form AdoCbl. *CD320* encodes a transcobalamin receptor that binds TCN2-cobalamin at the plasma membrane and internalizes the complex by endocytosis.

Combined MMA and homocystinuria is a genetically heterogeneous disorder of cobalamin (cbl; vitamin B12) metabolism. The defect causes decreased levels of the coenzymes adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl), which results in decreased activity of the respective enzymes methylmalonyl-CoA mutase and methyltetrahydrofolate–homocysteine methyltransferase, also known as methionine synthase. Different forms of the disorder have been classified: cblC, cblD, cblF, cblX, cblJ. CblC type is caused by mutation in the *MMACHC* gene on chromosome 1p34. The MMACHC protein may act as an intracellular cobalamin-trafficking chaperone and has been shown to act, in part, to catalyze the reductive decyanation of cyanocobalamin, generating cob(II)alamin, which is the substrate for assimilation into the active cofactor forms methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl). CblD type is caused by mutation in the *MMADHC* gene on chromosome 2q23, which plays a role in directing cobalamin to the 2 cobalamin-dependent enzymes, Methylmalonyl CoA mutase and Methionine synthase. CblF type is caused by mutation in the *LMBRD1* gene on chromosome 6q13. cblX type is an X-linked recessive metabolic disorder, caused by mutation in the *HCFC1* gene on chromosome Xq28, mutations of which inhibit its function in the transcriptional activation of *MMACHC* (*7*). cblJ type is caused by mutation in the *ABCD4* gene on chromosome 14q24, which is involved in the lysosomal release of cbl into the cytoplasm. Finally, deficiency of the enzyme methylmalonyl-CoA epimerase and ADP-forming succinyl-CoA synthetase caused by mutation of the *MCEE* gene on chromosome 2p13.3 and mutation in the *SUCLA2* gene on chromosome 13q14.2 can lead to benign subtypes.

The expression of miR-9 was recently found to be significantly down-regulated in MMA patient plasma and sensitively changed after VitB12 treatment, and up-regulation of miR-9 reduced neural apoptosis induced by methylmalonate via targeting BCL2L11. The above results show that miR-9 may act as a potential "competitor" of gas chromatography-mass spectrometry for the diagnosis of MMA and a monitor of changes in MMA and might provide new insights into a therapeutic entry point for treating MMA (*8,9*).

3. Diagnosis

Acidosis, ketosis, hyperammonemia, hypoglycemia, hyperglycemia, and neutropenia are main symptoms of MMA. Major secondary complications of MMA include developmental delay (variable), tubulointerstitial nephritis with progressive renal failure, "metabolic stroke" (acute and chronic basal ganglia involvement), disabling movement disorder with choreoathetosis, dystonia and para/quadriparesis, pancreatitis, growth failure, functional immune impairment, and optic nerve atrophy.

Combined with the above clinical features, MMA needs to be diagnosed by some laboratory methods. The basic laboratory markers suggestive of MMA include low bicarbonate levels less than 22 mmol/L in infants and less than 17 mmol/L in neonates, ketones in the urine, blood ammonia levels greater than 150 µg/dL in neonates, 70 µg/dL in infants, and 35-50 µg/dL in older children and adults, blood glucose levels less than 40 mg/mL in infants and less than 60 mg/mL in children, and absolute neutrophil counts less than 1,500/mm^3. Also, C3 values greater than 7 µmol/L and C3:C2 ratios greater than 0.2 measured by tandem mass spectroscopy (MS/MS) show suspected disorders of cobalamin or propionate metabolism. Finally, relatively high concentrations of methylmalonic acid and methyl citrate in urine from patients' gas chromatography/mass spectrometry (GC/MS) can lead to definitive diagnosis of the disorder.

Plasma homocysteine can be measured to identify gene types involved in MMA. Patients with very high concentrations of methylmalonic acid in urine, but normal homocysteine, have mutations in at least one

Table1. The hot mutation sites of Chinese patients

Number of Patients	Disease type	Pathogenic gene	Frequent mutation site	*Ref.*
16	cblC	*MMACHC*	c.609G>A /c.658_660delAAG	(*12*)
43	isolated MMA	*MUT*	c.729_730insTT	(*10*)
79	cblC	*MMACHC*	c.609G>A, c.658_660delAAG, c.482G>A, c.394C>T, c.80A>G	(*11*)

of the MUT (mut-, mut 0), cblB, cblA and cblD (var 2) subtypes. Patients with abnormally high concentrations of methylmalonic acid in urine and homocysteine in plasma have mutations in at least one of the cblC, cblF, or cblD (var 1) subtypes. Patients with slightly elevated methylmalonic acid in urine, but normal homocysteine, have mutations in at least one of the MCEE, SUCLA2 and benign MMA subtypes.

Mutation analysis is not only the gold standard diagnosis of MMA but also can aid in the choice of treatment strategy, B12 responsive or unresponsive. Several studies reported the gene mutation spectrum in Chinese patients with isolated MMA and cblC type MMA. The frequent mutation sites are presented below (Table 1). c.729_730insTT was the most frequent mutation among the 43 recruited Chinese isolated MMA patients (*10*). c.609G>A, c.658_660delAAG, c.482G>A, c.394C>T, c.80A>G accounted for 80% of disease alleles in 79 unrelated Chinese cblC type MMA patients (*11*). It was also reported by another recent study that c.609G>A and c.658_660delAAG were the most common mutations detected in 13 (81%) out of 16 patients (*12*).

Timely diagnosis and adequate treatment greatly improve the prognosis. In the past 15 years, many Chinese patients with this condition have achieved favorable treatment outcomes, and some of them have reached childbearing age. In 2015, the first case of a Chinese woman with cblC was reported to experience a successful pregnancy and deliver a healthy boy (*13*).

4. Treatment

Since the long-term neurodevelopmental outcome is strongly influenced by the duration of coma and peak blood ammonia concentrations, therapy must not be delayed and therefore the diagnostic workup and the initial medical treatment should proceed simultaneously: stabilize the patient; stop protein intake; start intravenous glucose; and seek expert metabolic advice. While waiting for the laboratory diagnosis, drugs including L-carnitine, hydroxocobalamin,biotin,sodium phenylbutyrate, l-arginine-Hcl and N-carbamyl-glutamate should be properly used.

Standard therapy of long-term management includes: L-carnitine; antibiotics to reduce intestinal flora; vitamin B12 in responsive MMA patients; low-protein diet; precursor-free amino acid and/or isoleucine/valine supplementation; vitamin and mineral supplementation; caring for special situations and provision of an emergency regimen in recurrent illnesses (*14*). Generally, cblc type is almost all B12-responsive, mut type is B12-unresponsive, and other types is partly responsive to B12. B12-responsive cblC type MMA is most common in China.

The use of specialized amino acid formulations containing minimal to no valine, isoleucine, methionine and threonine in the treatment for MMA has become widely implemented. However, two recent follow-up studies found that the excessive use of medical foods, especially in the setting of reduced natural protein intake, resulted in iatrogenic amino acid deficiencies and was associated with poor growth outcomes in a large cohort of isolated MMA and cblC type MMA patients (*15,16*). So, it is proposed that medical foods and dietary guidelines for MMA should be revised based on well-controlled and sufficiently powered clinical studies to support their efficacy and safety.

Because of the poor prognosis despite of the above treatment, centers started to pursue elective liver and combined liver-kidney transplantation as a treatment for the metabolic instability that eventually causes death since 1990s (*17*). Solid organ transplantation can eliminate the frequent episodes of metabolic acidosis, but has numerous practical limitations that include procedural availability, surgical mortality and morbidity, expense, a finite donor pool, and the need for life-long immune suppression. End stage renal disease (ESRD) is a cardinal manifestation of methylmalonic acidemia (MMA) Patients and MMA patients who have been treated by orthotopic liver transplantation. $Mut^{-/-}$;$Tg^{INS\text{-}Alb\text{-}Mut}$ mice model had been established to suggest that proximal tubular mitochondrial dysfunction is a key pathogenic mechanism of MMA-associated kidney disease, and identified lipocalin-2 as a biomarker of increased oxidative stress in the renal tubule, defining an approach for the treatment and monitoring of kidney disease in patients with MMA (*18*).

Due to limitations of organ transplantation, researchers studied viral gene therapy as treatment for MMA, using preclinical cellular and animal models since 2007. The treated $Mut^{-/-}$ mice have near-normal long-term survival and growth parameters, demonstrate enzymatic activity longer than one year after a single treatment with an AAV8 or AAV9 vector (*19,20*). Although genotoxicity was observed in the mouse studies with some vectors, it has been demonstrated that manipulating regulatory elements and AAV dosing could reduce the genotoxicity (*21*). Lack of neutralizing

Table 2. The reported epidemiological data of MMA patients in China

District	Time period	Number Screened	Number of Patients	incidence	Disease subtype	*Ref.*
Beijing,Shanghai	Before 2011	400,000	15	1:26,000	unknown	(*25*)
Shandong province	May 2011 to May 2014	35,291	9	1:3,920	cblC	(*26*)
Southern China	January 2009 to March 2012	16,075 with suspected inherited metabolic diseases	48	0.3%	Unknown	(*27*)
Throughout all the provinces of mainland China and Macao, with the exception of five provinces (Hainan, Neimenggu, Tibet, Ningxia, and Hong Kong)	February 2002 to June 2012	18,303 with suspected inherited metabolic diseases	242	1.32%	Unknown	(*28*)
Taiwan	August 2013 to December 2014	44,639	4	1:11,160	cb1C/mut	(*29*)
Henan Province	January 2013 to March 2016	349,858	58	1:6,032	Unknown	(*30*)

antibodies against AAV in MMA patients shown in a recent study (*4*) allow for the potential clinical application of systemic *AAV* gene delivery as a new treatment for mut MMA.

5. Epidemiological data of MMA patients in China

Estimates of incidence for MMA is reported to be 1/50,000 in Japan (*22*), 1/250,000 in Germany (*23*), and 1/85,000 in Taiwan of China (*24*).

MMA was first described in mainland China in 2000 (*25*). Along with the wide application of tandem mass spectrometry and gas chromatography/mass spectrometry, newborn screening for this metabolism error have been available in most parts of China. Some statistical results had been reported on the basis of screening data in different districts of China. As is shown below (Table 2), 15 MMA patients of the screened 400,000 babies before 2011 were diagnosed in Beijing and Shanghai, that is, the estimated incidence is 1:26,000 (*25*); 9 MMA patients were identified among 35,291 newborns in Shandong province screened between May 2011 and May 2014, giving an estimated incidence of 1:3,920 live births for MMA, and all were classified as cblC disease (*26*); in southern China, 16,075 urine samples were collected from patients who were highly suspected of having inborn errors of metabolism, among which 48 MMA patients were detected (*27*). Similarly, 242 MMA patients were confirmed in 18,303 patients with suspected inherited metabolic diseases throughout all the provinces of mainland China and Macao, with the exception of five provinces (Hainan, Neimenggu, Tibet, Ningxia, and Hong Kong) (*28*). 4 MMA patients were detected in 44,639 newborns from Taian district of China, and the estimated incidence is 1/11,160 (*29*); the reported prevalence of MMA in Henan province was 1/6,032, according to 349,858 screening results of newborns from January 2013 to March 2016 in this province (*30*).

Overall, cblC type is the most common type in Chinese patients. The data differ significantly in different districts of China, which may be related to the onset date of MMA screening. Additionally, the screening bases used by the above research vary. Specifically, the screening bases of two statistics were the number of patients with suspected inherited metabolic diseases in southern China and throughout all the provinces of mainland China and Macao with the exception of five provinces (Hainan, Neimenggu, Tibet, Ningxia, and Hong Kong), while the other statistical data are newborn screening results.

6. Conculsion

In conclusion, MMA is a severe genetic disease with poor prognosis. It can be defined by MS/MS and GC-MS. However, the importance of mutation analysis should lead to more sufficient awareness, which will guide the choice of treatment strategy. The efficacy and safety of low-protein diet for treatment of MMA should be reconsidered because of poor growth outcomes. Also, liver transplant, kidney transplant and combined liver-kidney transplant have been reported for the treatment of methylmalonic aciduria. However, organ transplantation for MMA remains controversial because the criteria for solid organ transplantation in MMA have not been well established and the high risk of complications. Viral gene therapy has been successfully tried at the animal model level, and will be a new potential treatment for mut MMA.

The reported epidemiological data on MMA in China was limited in local districts and the results differ significantly among them. The lack of national epidemiological data on MMA in China, which has been reported in other countries, may hinder improvement in healthcare for MMA. So, it is urgent to obtain accurate

data all over China based on the application of newborn screening strategy.

Acknowledgements

This work was supported by the Shandong Provincial Natural Science Foundation Program, China (ZR2015YL043) and the Innovation Project of the Shandong Academy of Medical Sciences.

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(Received March 19, 2018; Revised May 22, 2018; Accepted May 23, 2018)

Review

DOI: 10.5582/irdr.2018.01039

Current molecular insight to reveal the dynamics of CAG repeating units in spinocerebellar ataxia

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Summary **Spinocerebellar ataxia (SCA) is a heterogeneous genetic disorder with overlapping clinical phenotypes arising from the degeneration of purkinje cells and other regions of the brain. There are approximately 36 different subtypes of SCA, but SCA 1, 2, 3, 6 and 7 are most prevalent in the Indian population. Many findings suggested that cerebellar Purkinje cells region may be a uniquely vulnerable neuronal cell type, and more susceptible to a wider variety of genetic or cellular problems than other neuron types. In this review we emphasized mainly five common subtypes of SCA (1, 2, 3, 6 and 7) their pathophysiology, therapeutics, drugs studies and the technical challenges in the field of molecular genetic diagnosis.**

Keywords: Spinocerebellar ataxia, SCA, Triple Primed PCR (TP-PCR), polyglutamine disease, Autosomal Dominant Cerebellar Ataxia (ADCA)

1. Introduction

Spinocerebeller ataxia (SCA) is a rare, autosomal dominant, neurodegenerative disorder. SCA is mainly caused by a triplet repeat expansion and sometimes repeats can be pentanucleotide and hexanucleotide, however SCA can also be caused by point or missense mutations (*1*). Until now 40 different subtypes of SCA have been reported. Though the disease is mostly adult onset a few subtypes of childhood onset are also seen (*2*). SCA including subtypes 1, 2, 3, 6 and 7 are cause by the triplet Cytosine Adenine Guanine (CAG) repeat expansion in the precise genes that leads to the formation of an abnormally long polyglutamine chain in the respective encoded proteins (*3*).

SCA affects brainstem, spinal cord, cranial nerve nuclei, and cerebellum that finally lead to the progressive cerebellar ataxia, gait disturbance, nystagmus, dysarthria, tremor and ophthalmoparesis. Cerebellar degeneration does not only lead the movement problems but it also increases the variability and unbalancing of the whole body movement (*4-6*).

Clinical diagnosis of SCA subtypes is challenging because of the coinciding clinical features in different subtypes. To overcome these issues ADCAs (Autosomal Dominant Cerebellar Ataxia) classification of SCAs includes three major categories, Based on inheritance pattern and clinical features (*7*). Later on, the classification of ADCAs was little modified by Duenas *et al*. (*8*), on the basis of neuropathological features that provide a suitable characterization in clinical practice to facilitate genetic diagnosis. These categories are ADCA I, ADCA II and ADCA III. In ADCA I category SCA subtypes in which neuro-degeneration takes place just outside the cerebellum region, similarly for ADCA-II and ADCA-III neurological features with retinal degeneration and degeneration is only restricted to the cerebellum region respectively because degeneration is only restricted to cerebellum region for ADCA III category called pure form of the ataxia. The paternal transmission is mostly associated with the occurrence of repeat expansions in the next generation rather than maternal transmission of the expanded allele (*9*). The duration of disease is 10-15 years after onset. Now in this review we will discuss the five common subtypes of SCAs.

Released online in J-STAGE as advance publication May 23, 2018.

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2. Subtypes (SCA 1, 2, 3, 6 and 7) and their pathophysiology

2.1. *SCA1*

First case of SCA 1 was reported in the year 1993. SCA 1 appears in mid-30s and is characterized by gait disturbance, dysarthria and ocular Dysmetria which progresses to ophthalmoplegia (*10*). It is caused by CAG repeat expansion in coding region of *ATXN1* gene that encodes Ataxin-1 protein. The trinucleotide CAG repeat size for normal (unaffected), intermediate and affected alleles are ≤ 36, 36-44 and ≥ 44 respectively. Cytosine Adenine Thymine (CAT) interruptions (1 to 3 in no.) may be present in normal individuals, but in affected individuals the expanded repeats are present continually (*4*).These interruptions provide repeat size stability by avoiding the expansion of CAG repeats during (Deoxyribonucleic Acid) DNA replication (*11,12*). The mutated Ataxin-1 protein has an unusually long stretch of polyglutamine (CAG repeat encoded) that changes its 3D structure leading to abnormal interaction and aggregate formation with other nuclear proteins.

These aggregates decline to allow accurate functioning, which finally damages the purkinje cells, and finally leads to cell demise or degeneration of brain cells. Cytoplasmic localization unlike normal protein remains in the nucleus, so the problem is in cerebellar Purkinje cells that leads to progressive degeneration of the Purkinje cells.

The expanded *ATXN1* gene's alleles are similarly translated into proteins of apparently normal stability and distribution. The aggregates of protein are found only in the brain and spinal cord (CNS) region of the brain (*3,13*). Therefore, the cells within the cerebellum are very sensitive particularly to the alteration in ataxin-1 protein shape, and loss of cells of the cerebellum (Table 1).

2.2. *SCA2*

SCA2 first case was reported by Wadia and Swami and therefore this subtype also is called the Wadia-Swami Syndrome. It is the most prevelent form of SCA in the Indian population and is characterized by progressive cerebellar ataxia, Nystagmus and slow saccadic eye movements appears mostly in the 4th decade of life. It is caused by CAG repeat expansion in coding region of the *ATXN2* gene that encodes Ataxin-2 protein which is involved in cytoplasmic (Ribonucleic Acid) RNA-related functioning (*14-16*). The CAG repeat size range for unaffected individuals is ≤ 31 with (Cytosine Adenine Adenine) CAA interruptions while affected individuals, have an expanded uninterrupted region of more than 33 CAG repeats (*17*). The presence of CAA interruption does not ease the severity of pathogenicity as it also encodes for glutamine like CAG (*18*); yet

Table 1. Genetics of SCAs subtypes

Subtype	Chromosome Location	Gene	Exon (Mutation present)	Age onset	Distinguishing features (Including Ataxia)	Triplet Repeat	Repeat Size (Normal)	Repeat Size (Expanded)	Protein	Protein Location	Duration of disease (Years)	Affected Brain region
1	6	*ATXN1*	8	3rd – 4th decade	Active Reflexes	CAG	6 - 36	44 - 83	Ataxin 1	Nucleus & cytoplasm	15	Cerebellum
2	12	*ATXN2*	1	3rd – 4th decade	Slow saccadic eye movement	CAG	15 - 31	34 - 220	Ataxin 2	cytoplasm	10	Cerebellar purkinje cells
3	14	*ATXN3*	10	3rd – 4th decade	Muscle weakness and atrophy	CAG	12 - 40	54 - 86	Ataxin 3	Nucleus & cytoplasm	10	Ventral pons and Substantia nigra
6	19	*CACNA1A*	49	5th – 6th decade	Very slow progression of disease	CAG	4-18	21 - 33	Alpha-1A calcium channel protein	Membrane associated	> 25	Cerebellar purkinje cells
7	3	*ATXN7*	5	3rd – 4th decade	Visual loss	CAG	4-19	33 - > 300	Ataxin 7	Nucleus and cytoplasm	20	Cerebellar purkinje cells, Brain stem, Spinal cord

these interruptions may enhance the meiotic stability of the repeats (*19*). Previous studies have depicted that the absence of CAA interruption in expanded alleles may increase its instability leading to elevated risk of transmission of a larger expansion in the next generation. The affected Ataxin-2 protein finally targets the different pontine region and Purkinje cells in the cerebellum, and this protein is confined to the RNA containing stress granules, which is related to the endoplasmic reticulum/Golgi segment and plays a very significant role in cytoplasmic RNA-related functions (*14-16*) (Table 1).

2.3. *SCA3*

It is the most common subtype with clinical features, such as progressive ophthalmoplegia, ataxia, basal ganglia symptoms, pyramidal signs, dystonia, dysarthia and distal amyotrophies (*20,21*). It was first discovered in the family of Machado and Joseph and therefore this subtype is also called Machedo Joseph Disease or *MJD*. Normal repeat size for this subtype is ≤ 44 intermediate range and the mutated repeat range is 44 to 52 and 60-84 CAG repeats respectively. The SCA 3 causing gene is *ATXN3* that encodes the Ataxin3 protein. Over repetition of CAG in the *ATXN3/MJD1* gene ultimately translates into an affected Ataxin-3 protein causing neurotoxicity that might develop because of proteolysis of the main protein to liberate the expanded repeat fragment. The aggregation of proteins that is the hallmark feature of this subtype and also in this subtype Calpains (calcium-dependent cysteine proteases) initiates toxic fragment formation and ultimately leads to neuronal loss or degeneration (Table 1).

2.4. *SCA6*

It is also a common subtype of SCA and is characterized by cerebellar ataxia, dysarthria, and Nystagmus, with very slow progression that is the hallmark feature of this subtype (*22*). The CAG repeat ≤ 18 reported as the normal repeat size and 19 repeats is considered as the intermediate repeat size. In this subtype the repeat expansion mutation in the *CACNA1A* gene that encodes the $Ca_v2.1$ calcium channel protein. This gene has two splice site forms, Q-type and P-type isoforms and the CAG repeat expansion falls mainly in the P-type. This type also affects cerebellum where Purkinje cells are found within the Purkinje cell layer. In SCA6 affected individuals the Purkinje cells in, mutant $Ca_v2.1$ proteins form ovular intracellular inclusions. In study of cell culture models of this disease it presented early apoptotic cell death. Voltage-dependent calcium channels are the hetero-oligomeric proteins that comprise pore-forming α1 and auxiliary β, α2 and δ and in some tissues γ subunits (*23,24*).

As these calcium channels facilitate the entry of calcium into the cells in response to change in membrane potential so the disturbances in the Voltage-dependent calcium channels cause a number of neurological difficulties, such as epilepsy, migraine and cerebellar ataxias (*23,24*). An expanded CAG repeat in the *CACNA1A* gene results in a lengthened polyglutamine tract in the C-terminal region of the α1A subunit of *CACNA1A* protein. The heterologous expression of mutated α1A subunits enhances calcium channel deregulation, and finally interferes with the calcium homeostasis in Purkinje cells (Table 1).

2.5. *SCA7*

It is the fifth most common subtype of SCA with pigmented retinal atrophy as a distinguishing feature. The progression of this subtype is often more rapid and aggressive in children than adults. The clinical diagnosis is a little difficult in newborns because the ataxia and visual loss are not a very obvious symptom in SCA7 and failure to thrive and loss of motor milestones may be the initial symptoms (*25*). SCA7 causing gene *ATXN7*, is a polymorphic CAG repeat tract that falls in the first exon, while the normal allele size is between 4 to 19 and abnormal allele size is ≥ 37. The encoded protein is a component of SPT3/TAF9/GCN5 acetyl transferase (STAGA) and TBP-free TAF-containing (TFTC) chromatin remodeling complexes, and it plays an important role in the transcription regulation process. The process of Protein formation in affected individuals detected in the nuclear fraction appears to be ~130KD in size (*26*). The CAG repeat expansions in *ATXN7* decreases the transcription of an antisense non-coding RNA that promotes the repressive chromatin modification of the ataxin-7 promoter region (*27*) that leads to an increase in expression. Normal allele size is ≤ 36 and pathogenic allele size is ≥ 450 CAG repeats. Expansion mutation in *ATXN7* gene suppresses the transcription of an antisense non-coding RNA that promotes the repressive chromatin modification of the ataxin-7 promoter that leads to over expression of mutated protein (Table 1).

3. Genetics

It is a rare genetic disorder and the inheritance pattern is autosomal dominant. The CAG repeat expansion in the particular gene that present at specific locations of the chromosome and that gene codes for a particular amino acid, glutamine. Expansions cause the formation of glutamine expanse or polyglutamine tract. In this disease the gene involved in the formation of ataxin-1, ataxin-2, ataxin-3, ataxin-7 for SCAs 1, 2, 3, 7 respectively and the location of these genes is 6p, 12q, 14q, 3p respectively and for the SCA 6 subtype gene is *CACNA1A* and location of this gene is 19p (Table 1).

The proteins encoded by these genes is involved in

destroying and getting rid of the surplus, damaged or unneeded proteins that presents in the cells. The role of Ataxin proteins is to eliminate the ubiquitin from these unwanted proteins just before they are ready for degradation so that the ubiquitin can be used again. Ataxin proteins are also involved in regulation of the first stage of protein formation (transcription). In the case of SCA6 because of the expansion mutations in the *CACNA1A* gene, encodes for the protein that acts as a pore forming α1A subunit of P/Q type calcium channels and is responsible for starting and regulation of synaptic transmission (*28*).

4. Diagnosis

Diagnosis of Spinocerebellar ataxia is primarily based on the clinical characteristics and the next need is for evidence of family history. Like many diseases with known genetic causes, a family history that can disclose multiple family members with similar clinical conditions can easily indicate the diagnosis of SCA. However, an ataxic patient whose family history constitutes a genetically confirmed diagnosis of a spinocerebellar ataxia subtype is a perfect candidate for genetic testing, but such types of cases are not very constant. If the movement related problems existed previously in the family record, a previous diagnosis is likely to show a classification given to the disease at the time of diagnosis. So the diagnosis of SCA divided into the two types first is clinical diagnosis and second is molecular genetic diagnosis.

4.1. *Clinical diagnosis*

For clinical diagnosis if the patient presents ataxic features like movement problems, Nystagmus, dysphagia, dysarthia *etc*. then there is a requirement to check this through the help of some neurological testing like CT scanning and Brain MRI (*29*), because in SCAs degeneration of cerebellum is present. So if shrinkage of cerebellum is manifested in CT scanning and Brain MRI then next molecular confirmation and subtyping of SCA needs to continue for molecular genetic diagnosis.

4.2. *Molecular diagnosis*

The molecular diagnosis of SCAs relies on the tests that determine the number of the triplet (CAG) repeat elements in the particular gene. Southern blot analysis was the first method but It has some drawbacks because this method is expensive, time consuming, radioactive based and requires a large amount of DNA concentration for a single reaction. Polymerase Chain Reaction (PCR) was used to detect the triplet repeat expansions that are less than ~100 repeat (*30*), the PCR technique can reveal the pattern of alleles either two heterozygous peaks of normal-sized alleles or a single homozygous peak normal-sized. When the repeat expansions number more than 100 repeats, or fall outside of the detectable range by PCR and would require Southern Blot analysis or triplet primed PCR, for routine testing of expansion mutations you can usually perform either Southern blot analysis (*31,32*) or a long-range PCR method (*33,34*). However long-range PCR is cost effective and much faster than southern blotting.

Short PCR method for routine diagnosis of the larger triplet repeat mutations in Indian Triplet repeat disorder patients is recommended as a best method for molecular diagnosis for Triplet repeat disorders (TRDs) in a very fast and cost effective manner. By Normal or Short PCR analysis we cannot detect the repeat expansion of ≥ 100 repeats so in such a case a useful method is TP-PCR (triple primed PCR), to detect triple repeat expansion in cases of more than 100 repeats. TP-PCR (Triplet repeat primed PCR) is a new and advanced technique to detect large triplet (CAG) repeat expansion mutations in (Mytonic Dystrophy) DM1 and SCA patients (*35*). In the Indian scenario previously many studies have been described roughly about the usefulness and benefits of the TP-PCR method for quick diagnosis and proper genetic counseling in triplet repeat disorders (*36*). TP-PCR is very beneficial explicitly for the uncovering of repeated expansion mutations.

5. Treatment and management

Inopportunely, there is no proper treatment available for SCAs but for management help using cane sticks and walkers may be helpful to prevent falls of ataxic patients, and prepare ramps for mechanized chairs. Generally, treatments are directed towards progression of the symptoms, not for the disease itself. Some therapies and medications might be appropriate for some of these particular symptoms, like depression, spasticity, tremors, sleeping problems and some others (Table 2).

Voxel-based morphometrics can also expose the volume loss in cerebellum and brain stem region involving both gray and white matter of the brain (*37,38*). The region degeneration may also be able to be seen by this method (*39*). Some other landmarks such as the measurements of some metabolites such as myo-inositol and -acetyl aspartate that can disclose the evidence of neuronal cell loss in the pons and cerebellum and even the supratentorial structures of brain (*40*). Loss of cerebellar and brain stem grey matter and motor dysfunction problems disclosed by quantitative imaging studies have been recently documented in pre-symptomatic persons known to have an *ATXN1* triplet repeat expansion mutation.

A number of therapies and educating places should be available for ataxic and dysarthria patients. These therapies should include, phonological therapy, writing

Table 2. Main symptomatic treatment proposed for patients with autosomal dominant hereditary ataxias

Symptomatic treatment (Drugs)	Ataxia type
Riluzole 100 mg/day	SCAs and other etiologies (recessive and sporadic)
protirelin tartrate or taltirelin hydrate	SCA1
protirelin tartrate or taltirelin hydrate	SCA2
Varenicline 1 mg twice day	SCA3
Buspirone 30 mg twice daily	SCAs
Oral zync 50 mg/day	SCA3
Insulin-like growth factor-1 A	SCA3
Mexiletine and Carbamazepine	SCA3 (pain and cramps)
Botulinum toxin type A	SCA3 (dystonia and spasticity
protirelin tartrate or taltirelin hydrate, Acetazolamide, gabapentin and pregabalin	SCA6
protirelin tartrate or taltirelin hydrate	SCA7

therapy, speech therapy and rehabilitation, different work-related therapies, dietetics essential to give them psychosomatic care and also support from social services. Towards the direction of treatment with the help of therapies should be communication devices, should train patients to carry their eating utensils and also have dressing hooks for help to make them self-dependent and can explain coordinative physiotherapy. Many SCA patients have other symptoms, in addition with the ataxia so some medications and some other therapies might be helpful for these symptoms. The noxious protein oligomers potentially can be reduced through proper stimulation of Heat Shock Protein (Hsp) members, although reducing the level of transcriptional deregulation and RNA aggregation may also be helpful in progression of disease. Compounds which can limit the intensity of the agitation of Purkinje neuron cells and reduce the level of release of intracellular calcium, have been established as advantageous in multiple model studies of different subtypes of SCAs. Compounds such as SK channel activating compounds and dantrolene, might recover the function of purkinje cells by adaptable pacemaker firing disturbances and could diminish the stimulation or activation of Ca^{2+} dependent cell death processes that ultimately leads to neuronal cell deterioration. Numerous clinical trial studies revealed the success of Riluzole drug and showed SK channel stimulation may have a particular beneficial effect for the purpose of treatment of several etiologies of SCAs.

Some neuro-protective medications or drugs (*N*-methyl-D-aspartate antagonists) are even now available in phase trials. So development of a reliable ataxia rating scale to screen disease progress and treatment responses has been started. Forthcoming convenience of the therapeutic interventions would slightly change indications of the DNA testing and its psychological and social impacts.

6. Genetic counseling and Preimplantation genetic diagnosis

As previously described its inheritance pattern is autosomal dominant means that if any patient's parents have a mutant allele, there is 50% chance to the sibs to inherit the mutant alleles. For this reason, many couples with affected parents mostly choose to not plan for a child in the future. For the last two decades developmental of Preimplantation genetic diagnosis (PGD) which contains testing of the fertilized ova (in vitro fertilization (IVF)) to distinguish mutation in the affected gene, and after implantation of selected particular healthy embryos to ensure that the presence of pathogenic alteration from parents will not be transmitted to the next generation (*41*). The genetic test must be achieved in respect to formal genetic counseling. This testing is not very beneficial in estimating the severity, age of onset, symptoms types or rate of progression in individuals who are asymptomatic.

7. Prevalence

The prevalence of SCA with great accuracy is a very difficult task to clarify because in most of the studies it is explained various ways. First subtype of SCA is reported from diverse ethnic groups worldwide with varying prevalence, SCA1 estimated prevalence is 22% of total ADCAs (Autosomal Dominant Cerebellar Ataxia) in India. So the prevalence of some late onset SCAs may be underestimated, however, on the basis of available reported studies, SCA different subtypes such as 1, 2 and 3 accounts for the most prevalent in the whole world's population. SCA 3 subtype first originated from two families of the MJDs, who were the Azorean descents found in different ethnic populations and were found to be the most common in different countries like Germany and US. SCA2 subtype is the most common subtype in countries like southern Italy, Spain, Cuba and India. Dentatorubral - pallidoluysian atrophy (DRPLA) is most reported from Japan and is very rare in North America, so it is most prevalent in Japan (*42-51*). Another scientist Soong and his colleagues reported SCA3 was the most common subtype of Autosomal Dominant cerebellar ataxia (ADCA) in Taiwanians (47.3%), next followed by subtype SCA6 (10.8%), SCA2 (10.8%), SCA1 (5.4%), SCA7 (2.7%), SCA8 (2.7%), and DRPLA (1.4%) and

rare in Indians.

In another study from Singapore, scientists Zhao *et al*, reported that prevalence of this disease among Singaporean populations was about 1 out of 27,000 population (*52*). Researchers observed a founder effect for the specific subtypes of SCA, On the basis of history and ancestry of spinocerebellar ataxia (SCA), as well as the correlation between subtypes and ethnicity. So the global prevalence of SCAs fluctuates from 0.3 to 3.0 per 100,000 population (*53*).

8. Conclusion

Numerous SCA causing mutations are currently known, nonetheless there are many other mutations that remain unknown. So the target for convergent mechanisms of neuronal dysfunction in Ataxia needs to be a most effective therapeutic mediation in the near future. It has been more than one decade since the discovery of SCA but the disease continues to hold surprises in spite of extensive research in this field. Among the primary goal of the researchers, it is to find effective targeted therapy for this disease and also to develop speedy, sensitive and cost effective genetic diagnostic methods. In present review, all the insight of genetics, treatment, medications, therapies and diagnosis of the five most common subtypes of SCA is discussed. More research is required to reveal the precise drugs and proper treatment for designing and validating possible drug targets. Succeeding advancement in therapy, early recognition of the disease is also a big concern. The early molecular diagnosis of SCA is too important since to ensure not only affected persons and also their members who can receive all possible benefits through genetic diagnosis, including genetic counseling that is very important for prenatal diagnosis for the risk of recurrence of SCAs high in the family and their relatives.

TP PCR has preferably substituted traditionally used techniques owing to its sub sensitivity, selectivity, and very low cost. It offers the possibility of early diagnosis in clinical suspects, and prenatal testing. Progress in both genetic diagnosis and therapy would hopefully improve the quality of life for the SCA patients in the near future.

Acknowledgments

The author is thankful to University Grant Commission (UGC) (F.No. 19–1/2015(SA–I) – New Delhi for providing fellowship and Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow for Lab support.

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(Received April 20, Revised May 1, 2018; Accepted May 13, 2018)

Original Article

DOI: 10.5582/irdr.2018.01032

Generation of urine-derived induced pluripotent stem cells from a patient with phenylketonuria

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Summary **The aim of the study was to establish an induced pluripotent stem cell line from urine-derived cells (UiPSCs) from a patient with phenylketonuria (PKU) in order to provide a useful research tool with which to examine the pathology of this rare genetic metabolic disease. Urine-derived epithelial cells (UCs) from a 15-year-old male patient with PKU were isolated and reprogrammed with integration-free episomal vectors carrying an OCT4, SOX2, KLF4, and miR-302-367 cluster. PKU-UiPSCs were verified as correct using alkaline phosphatase staining. Pluripotency markers were detected with real-time PCR and flow cytometry. Promoter methylation in two pluripotent genes, *NANOG* and *OCT4*, was analyzed using bisulphite sequencing. An embryoid body (EB) formation assay was also performed. An induced pluripotent stem cell line (iPSC) was generated from epithelial cells in urine from a patient with PKU. This cell line had increased expression of stem cell biomarkers, it efficiently formed EBs, it stained positive for alkaline phosphatase (ALP), and it had a marked decrease in promoter methylation in the *NANOG* and *OCT4* genes. The PKU-UiPSCs created here had typical characteristics and are suitable for further differentiation.**

Keywords: Phenylketonuria, induced pluripotent stem cells, urinary cells, integration-free, disease model

1. Introduction

The high molecular weight melanoma-associated Phenylketonuria (PKU, OMIM 261600) is a common inherited metabolic disease due to mutations in the phenylalanine hydroxylase (PAH) gene that cause accumulation of phenylalanine in the blood and brain, leading to multiple clinical manifestations. These manifestations are mainly development and mental retardation and occasionally include epilepsy, eczema, skeletal fragility, corneal softening, and congenital heart disease (*1,2*). Restrictions on the intake of phenylalanine are the main treatment for PKU, but the effectiveness of that treatment differs among individuals (*3,4*). More importantly, such strategies do not target the molecular pathways caused by mutations in the PAH gene, so they cannot reverse every symptom. Therefore, the pathology of PKU needs to be ascertained more accurately in order to identify more potential therapeutic targets (*5*).

Currently, study on the pathogenesis of PKU is limited, which is partly due to the lack of appropriate disease models, and in particular those that can mimic the pathology of the disease *in vitro* and *in vivo* (*1,6*). In clinical settings, obtaining cells associated with PKU, like neural cells, is also difficult.

The recent advent of induced somatic cell reprogramming with a combination of factors has allowed different disease-related cells to be obtained from induced pluripotent stem cells (iPSCs) (*7*), thus providing a new "disease model in a dish" strategy with which to study the biological characteristics of many diseases, and rare genetic disorders in particular (*8,9*). iPSCs can be differentiated into different types of cells and iPSCs obtained from patients share the

Released online in J-STAGE as advance publication May 15, 2018.

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same genetic background, so iPSC-based models can mimic almost the entire process of pathogenesis. These aspects explain why iPSCs have garnered considerable attention (*10-12*).

The current study developed a noninvasive and integration-free approach to generating PKU-UiPSCs from urine-derived cells, allowing a continuous supply of those cells for *in vitro* research on mechanisms and treatments of PKU.

2. Materials and Methods

2.1. *The patient with PKU and ethical approval*

Urine samples were collected from a 15-year-old male Han Chinese patient with PKU after informed consent was obtained. PKU was diagnosed during neonatal screening based on a blood Phe concentration of 22 mg/dL. Once the diagnosis was confirmed, the patient was subject to dietary restrictions to prevent any irreversible neurological damage. The patient has no significant symptoms at this time. This study was approved by the ethics committee of Shandong Medical Biotechnological Center.

2.2. *Culture of epithelial cells from urine*

Cells were isolated from urine using the method described by Zhou *et al*. (*13*). Briefly, 500 mL of urine was collected from the patient and then centrifuged at 400 g for 10 min. The supernatant was discarded, and the cells were washed with 10-15 mL of PBS containing 1% penicillin and streptomycin (Gibco, USA). Cells were centrifuged again and then resuspended in 3 mL of urine cell medium (1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) high glucose (Life Technologies, USA) with 10% (vol/vol) fetal bovine serum (FBS) (Gibco, USA), 1% 100× nonessential amino acid (Gibco, USA), 1% 100× L-GlutaMax (Gibco, USA) and Renal Cell Growth Medium (REGM) SingleQuot kit supplements (Lonza)). Cells were seeded in one well of a 6-well plate (Sorfa, China) coated with 0.1% gelatin (Millipore, USA). The medium was then removed and replaced with urine cell medium. Subsequently, half of the culture medium was replaced daily. Upon reaching 90% confluence, cells were passaged using Trypsin (Gibco, USA) with a split ratio of 1:2-1:3 and subcultured for a maximum of 3 passages.

2.3. *Reprogramming and iPSC culture*

Integration-free episomal vectors carrying the OCT4, SOX2, KLF4, and miR-302-367 cluster were used to perform reprogramming. Cultured cells at a density of 10^5-10^6 cells were electrotransfected (Lonza, Switzerland). Cells were seeded in 6-well plates coated with Matrigel (Coring, USA) in urine cell medium. After 48 h, the medium was replaced with IM1 medium (Osinglay, China), and cells were cultured for 10-16 d while replacing the medium every day until iPSC colonies appeared. Individual colonies were removed using a glass needle and expanded in Biociso medium (Osinglay, China) on plates coated with Matrigel. The iPSCs were passaged every 3-5 d using 0.5 mM EDTA (Life Technologies, USA) in DPBS (Gibco, USA) at a ratio of 1:3-1:5.

2.4. *Confirmation of the absence of the reprogramming vectors*

After 10 passages, iPSCs were tested for the absence of programming vectors. A real-time quantitative polymerase chain reaction (RT-PCR) was performed to detect vector genomes and transgenes. To that end, total DNA was isolated using a genomic DNA kit (Tiangen, China) in accordance with the manufacturer's instructions. RT-PCR was performed as suggested by the manufacturer of the Taq-HS PCR Forest Mix (Nova, China): 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and finally 72°C for 5 min. Primer sequences are shown in Table 1. PCR products were analyzed using 1% agarose gel electrophoresis.

2.5. *ALP staining*

The BCIP/NBT Kit (CoWin Biosciences, China) was used to perform ALP staining to preliminarily identify pluripotency. The cells in one well of a 6-well plate were washed twice with PBS and then 4% polyoxymethylene was used to fix the cells. Afterwards, the well was

Table 1. List of primers for vectors

Target genes	Primer sequence
OCT4	
Forward	AGTGAGAGGCAACCTGGAGA
Reverse	AGGAACTGCTTCCTTCACGA
SOX2	
Forward	ACCAGCTCGCAGACCTACAT
Reverse	CCCCCTGAACCTGAAACATA
KLF4	
Forward	CCCACACAGGTGAGAAACCT
Reverse	CCCCCTGAACCTGAAACATA
SV40LT	
Forward	TGGGGAGAAGAACATGGAAG
Reverse	AGGAACTGCTTCCTTCACGA
oriP	
Forward	TTCCACGAGGGTAGTGAACC
Reverse	TCGGGGGTGTTAGAGACAAC
EBNA-1	
Forward	ATCGTCAAAGCTGCACACAG
Reverse	CCCAGGAGTCCCAGTAGTCA
miRNA-302	
Forward	TTTCCAAAATGTCGTAATAACCCCG
Reverse	CTCCCAAAGAGTCCTGTTCTGTCCT

washed twice using TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20). A color reagent was then prepared according to the manufacturer's instructions and added to the well for 15 min.

2.6. *Detection of the pluripotency of markers*

Real-time quantitative PCR or RT-qPCR was used to analyze the endogenous pluripotency genes *OCT4* and *NANOG* in iPSCs. Quantitative PCR reactions were performed using 5 ng of reverse-transcribed cDNA with 5 μL of Sybr Green Realtime PCR Master Mix (TOYOBO, Japan) and the primers listed in Table 2. The cycle program was as follows: 95°C for 1 min, 45 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. Each reaction was run in technical triplicates on the Light Cycler®480 (Roche, Switzerland) and normalized to β-actin as an endogenous control. All data were calculated using the ΔΔCp method.

2.7. *Flow cytometry of cell surface markers*

Harvested iPSCs were resuspended in 100 uL of FACs buffer (PBS with 2% FBS). Antibodies (BD, USA) against nuclear transcription factor OCT3/4 and the surface markers TRA-1-60, TRA-1-81, and SSEA-4 as described in Table 3 were added in accordance with the manufacturer's instructions. Cells were incubated at 4ºC for 40 min. Permeabilization buffer was added after centrifugation. Cells were centrifuged for 1,500 rpm at 10 min and then resuspended in 100 uL of FACs buffer prior to sorting on the BD FACS Aria II (BD, USA).

2.8. *Bisulfite promoter sequencing*

A DNA Methylation-Direct Kit (EZ, USA) was used to perform bisulfite treatment in accordance with the manufacturer's protocol, and the partial sequence of the promoter region was amplified with nested PCR. The primer sequences are shown in Table 4. The products of amplification with PCR were ligated into a T-vector and cloned into DH5α bacteria. Ten clonal colonies were selected and sequenced.

2.9. *Formation of embryoid bodies*

Embryoid bodies (EBs) formed once UiPSCs reached 95% confluence. Cells were washed with DMEM/F12 (BI, Israel) and then PDE1 (Osinglay, China) was applied for 9 min. A fine-tip Pasteur pipette was used to draw grid lines and then collect cells. The cells were resuspended in EB differentiation medium. Cells were plated onto ultra-low attachment plates for 7 d. Medium was replaced every other day. On day 8, EBs were collected and then replated on Matrigel-coated

Table 2. List of primers for pluripotency and differentiation markers

Target genes	Primer sequence
OCT4	
Forward	CCTCACTTCACTGCACTGTA
Reverse	CAGGTTTTCTTTCCCTAGCT
SOX2	
Forward	CCCAGCAGACTTCACATGT
Reverse	CCTCCCATTTCCCTCGTTTT
NANOG	
Forward	AAGGTCCCGGTCAAGAAACAG
Reverse	CTTCTGCGTCACACCATTGC
Actin	
Forward	CCCAGAGCAAGAGAGG
Reverse	GTCCAGACGCAGGATG
FOXA2	
Forward	CCAACCCCACAAAATGGA
Reverse	ATAATGGGCCGGGAGTACA
SOX17	
Forward	ACCGCACGGAATTTGAAC
Reverse	GCAGTAATATACCGCGGAGC
BRACHYURY	
Forward	CCCTATGCTCATCGGAACA
Reverse	TTCCAAGGCTGGACCAAT
MSX1	
Forward	TCCGCAAACACAAGACGA
Reverse	ACTGCTTCTGGCGGAACTT
MAP2	
Forward	TGAAGCAAAGGCACCTCAC
Reverse	TATGGGAATCCATTGGCG
PAX6	
Forward	TTGCTTGGGAAATCCGAG
Reverse	TGCCCGTTCAACATCCTT
GAPDH	
Forward	GGTGGTCTCCTCTGACTTC
Reverse	CTCTTCCTCTTGTGCTCTTG

Table 3. List of antibodies for flow cytometry

Target	Antibody	Volume
TRA-1-60	PerCP-Cy™5.5 Mouse Anti-Human TRA-1-60Antigen	5 uL
TRA-1-81	FITC Mouse Anti-Human TRA-1-81 Antigen	20 uL
SSEA-4	Alexa Fluor® 647 Mouse anti-SSEA4	20 uL
OCT3/4	PE Mouse anti-Oct3/4	20 uL

Table 4. List of primers for bisulfite promoter sequencing

Target genes	Primer sequence
BSP-*OCT4-1*	
Forward	AGGTGTGGGAGTGATTTTAGATAGT
Reverse	AAACCTTAAAAACTTAACCAAATC
BSP-*OCT4-2*	
Forward	GAGGTTGGAGTAGAAGGATTGTTTTGG
Reverse	CCCCCCTAACCCATCACCTCCACCACC
BSP-*NANOG-1*	
Forward	TTGTTGTTTAGGTTGGAGTATAGTGG
Reverse	CCTAACGAACACACCCCCTACT
BSP-*NANOG-2*	
Forward	TGGTTAGGTTGGTTTTAAATTTTTG
Reverse	AACCCACCCTTATAAATTCTCAATTA

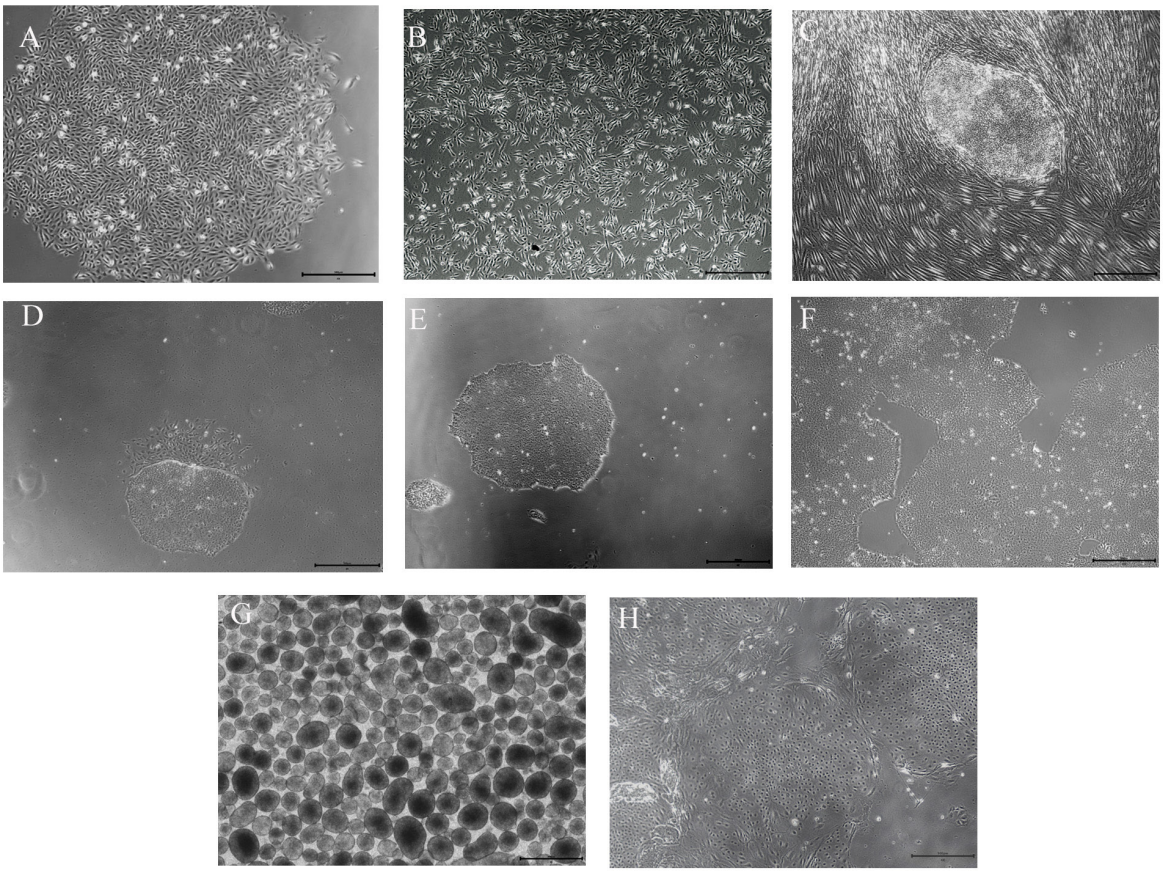

Figure 1. Main types of cell culture (Scale bar = 500 μm). (A) Morphology of urine cells. **(B)** Urine cells on day 3 after electrotransfection. **(C)** iPSC colonies. **(D)** iPSC colonies after transition to feeder-free conditions with differentiated cells. **(E)** Purified colonies of iPSCs. **(F)** Stable growth and rapid proliferation of iPSC colonies are evident. **(G)** EBs are suspended. **(H)** Change in the morphology of EBs with adherence.

6-well culture plates for 7 d. Afterwards, total RNA was extracted to detect 3 germ layers using RT-qPCR. The primer sequences are shown in Table 2.

3. Results

3.1. *Derivation of PKU-UiPSCs*

Integration-free episomal vectors with the OCT4, SOX2, KLF4, and miRNA 302-307 cluster were transfected into PKU urine cells (Figure 1A). The plasmid was added, and the cells began several stages of growth (Figure 1). After first day of electrotransfection, a large number of cells failed to adhere and died. On days 3-5 of electrotransfection, the cells grew stably, and the number of cells increased markedly (Figure 1B). After days 5-7, the medium contained numerous cells. On day 10, embryonic stem cell (ESC) like clones were evident (Figure 1C). On day 21, the clones reached confluence and matured. The clones were separated from surrounding cells with a glass needle and then transferred to a new culture plate (Figure 1D). After 3-5 passages, the differentiated cells gradually decreased until they disappeared (Figures 1E and 1F). Embryonic stem cell-like colonies were stably cultured and passaged.

3.2. *Verification of the absence of reprogramming vectors*

After purification, PKU-UiPSCs displayed typical phenotypes like ESCs. After 10 passages, reprogramming vectors were detected. As shown in Figure 2A, no exogenous genes were amplified in the established PKU-UiPSCs, indicating the integrity of iPSCs after reprogramming.

3.3. *ALP staining*

ALP was highly expressed in ESCs and iPSCs but slightly expressed or not expressed in differentiated cells. After staining, alkaline phosphatase-positive ESC-like colonies were evident because they stained distinctly blue while PKU urine cells were barely stained (Figure 2B).

3.4. *Detection of pluripotency markers*

Pluripotency markers (endo*OCT4*, endo*SOX2*, and

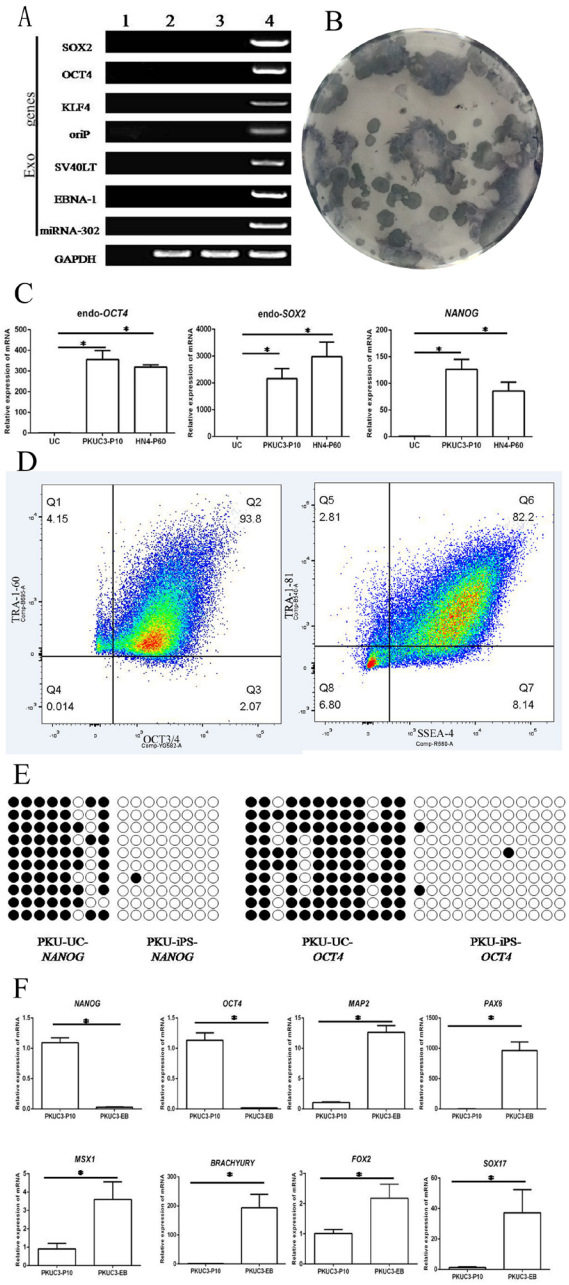

Figure 2. iPSCs from a patient with phenylketonuria are pluripotent. (A) Test for absence of reprogramming vectors. RT-PCR of PKU-UiPSCs after 2 passages in (4) the positive control, urine cells (UCs) in (2) the negative control, (3) PKU-UiPSCs after 12 passages, H_2O in (1). **(B)** Alkaline phosphatase-positive ESC-like colonies (blue) from the PKU UCs (white). **(C)** Pluripotent UiPSCs (PKUC3-P10) and ESCs (HN4P60) expressed higher levels of the pluripotency markers *OCT4*, *NANOG*, and *SOX2* in comparison to UCs ($p < 0.05$). **(D)** Flow cytometry of the pluripotency markers anti-stage specific embryonic antigen 4 (SSEA-4) (cell surface), anti-Tra-1-60 (cell surface), anti-Oct3/4 (nuclear), and anti-Tra-1-81 (cell surface). **(E)** Bisulfite genomic sequencing of the key pluripotent gene *NANOG* and *OCT4* promoter area indicated a marked decrease in methylation in UiPSCs. The open and closed circles represent unmethylated and methylated CpGs, respectively. **(F)** Differentiated cultures of 7-day-old EBs (PKUC3-EB) expressed higher levels of 3 germ layer markers and lower levels of pluripotency markers in comparison to UiPSCs (PKUC3-P10) ($p < 0.05$).

NANOG) were assayed in PKU-UiPSCs (Figure 2C). Expression of endo*OCT4* was 400-fold that in PKU urine cells. Expression of endo*SOX2* in PKU-UiPSCs was 3,000-fold higher, and expression of *NANOG* was about 150-fold higher. Expression differed little between PKU-UiPSCs and human ESCs.

3.5. *Flow cytometry*

Flow cytometry detected all of the markers in PKU-UiPSCs (Figure 2D). OCT3/4 and TRA-1-60 were detected in 93.8% of iPSCs (the Q2 region). In the Q6 region, about 82.2% of cells were positive for SSEA-4 and TRA-1-81.

3.6. *Bisulfite promoter sequencing*

Bisulfite genomic sequencing indicated that cytosine guanine dinucleotides (CpG) in the promoter regions of *OCT4* and *NANOG* were highly unmethylated in iPSCs in comparison to the urine cells from the same donor (Figure 2E). This indicates that the promoters were reactivated in iPSCs and that the urine cells were reprogrammed by reprogramming factors.

3.7. *EB formation*

iPSCs formed 3 germ layers in EBs (Figures 1G and 1H). Genetic markers of pluripotency and the 3 germ layers were detected with RT-qPCR (Figure 2F). Using PKU-UiPSCs from the same donor as negative controls, PKU-EBs had about a 5-1,000-fold increase in levels of markers of the endoderm (*FOXA2*, *SOX17*), mesoderm (*BRACHYURY*, *MSX1*), and ectoderm (*MAP2*, *PAX6*), RT-qPCR also revealed a considerable decrease in expression of pluripotency markers (*OCT4* and *NANOG*), indicating that iPSCs were devoted to developing the 3 germ layers.

4. Discussion

The induction of iPSCs provides a new tool with which to study rare diseases. The current study collected urine from a patient with PKU and it then obtained UiPSCs. Several experiments demonstrated the pluripotency of those PKU-UiPSCs. ALP staining revealed a high level of alkaline phosphatase expression. RT-qPCR and flow cytometry indicated that markers of pluripotency were highly expressed. Methylation sequencing revealed a marked change in methylation of the promoter region of pluripotent markers in UCs and UiPSCs. iPSCs were able to differentiate into all three germ layers *in vitro* through EB formation. In conclusion, PKU-UiPSCs were generated from urine-derived cells, and in principle the generated cells have the ability to differentiate into specialized cells and tissues.

Significant advances have been made in determining

the pathogenesis of many rare diseases, drug screening, and regenerative medicine as a result of studies using patient-specific iPSCs (*14-17*). In comparison to a previous study that generated PKU-iPSCs from peripheral blood mononuclear cells (PBMCs) (*16*), UiPSCs in the current study were generated from kidney epithelial cells in urine, and the latter approach has clear advantages (*18*). First, urine-derived cells can be generated less expensively than cells from other sources such as PBMCs and fibroblasts, and this generation is simple, consistent, and safe for researchers. Second, urine cells can be readily obtained from patients because ample urine is naturally excreted by the body and urine collection poses no burden to the patient. Third, UiPSCs have a high level of reprogramming efficiency (*19*). In short, UiPSCs are currently the best way to create a bank of PKU-associated cells with different types of gene mutations.

In addition, several studies have found that UiPSCs preferentially differentiate into neurons and that even epithelial-like cells from human urine can directly differentiate into neural progenitor cells (*20,21*). Although studies have suggested that irreversible nerve damage due to a high concentration of phenylalanine in the blood may be related to brain-derived neurotrophic factor (*22,23*), no studies have thoroughly examined the mechanism whereby hyperphenylalaninemia results in mental retardation. PKU-UiPSCs provide a useful approach with which to understand the neurological effects of PKU since UiPSCs differentiate into neurons and the ability to create key cell types not directly available from patients with PKU.

Moreover, the ability of iPSCs to differentiate into cells such as osteoblasts (*8*) and osteoclasts (*24*) means that PKU-UiPSCs could be highly useful in the study of the biological mechanisms underlying bone impairment in PKU (*25,26*). Disease models using UiPSCs from patients with PKU offer an unprecedented opportunity to develop new treatments such as enzyme substitution therapy using Phe ammonia lyase (PAL) before conducting clinical experiments (*27*) and to identify targets in order to correct PAH misfolding or to develop new therapeutic candidates (*28*).

The current study generated iPSCs from urine-derived cells from a patient with PKU and it verified the pluripotency of those cells. The UiPSC line could be used in advanced research and to provide insight into the clinical variability of the disease, the effects of genetic background, and epigenetic influences in humans.

Acknowledgements

This study was supported by the Innovation Project of the Shandong Academy of Medical Sciences & Key Projects in the National Science & Technology Pillar Program under the Twelfth Five-year Plan (2013BAI07B00).

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(Received April 8, 2018; Revised May 2, 2018; Accepted May 8, 2018)

Original Article

DOI: 10.5582/irdr.218.01038

Establishment of a human trisomy 18 induced pluripotent stem cell line from amniotic fluid cells

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Summary **Trisomy 18 (18T) is the second most common autosomal trisomy syndrome in humans, but the detailed mechanism of its pathology remains unclear due to the lack of appropriate models of this disease. To resolve this problem, the current study reprogrammed human 18T amniotic fluid cells (AFCs) into an induced pluripotent stem cell (iPSC) line by introducing integration-free episomal vectors carrying pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, pCXLE-hUL, and pCXWB-EBNA1. The pluripotency of 18T-iPSCs was subsequently validated by alkaline phosphatase staining, detection of iPSC biomarkers using real-time PCR and flow cytometry, detection of embryoid body (EB) formation, and detection of *in vivo* teratoma formation. Moreover, this study also investigated the transcriptomic profiles of 18T-iPSCs using RNA sequencing, and several gene clusters associated with the clinical manifestations of 18T were identified. In summary, the generated induced pluripotent stem cells line has typical pluripotency characteristics and can provide a useful tool with which to understand the development of 18T.**

Keywords: Trisomy 18, induced pluripotent stem cells, disease model, rare disease

1. Introduction

Trisomy 18 syndrome (18T, also Edwards syndrome) is the second most frequent autosomal trisomy syndrome in humans. This syndrome involves the presence of an extra chromosome 18 (*1*). The incidence of 18T is estimated to be 1/6,000-1/8,000 births (*2*). This trisomy usually manifests as abnormal development of multiple tissues and organs during prenatal ultrasound screening and is subsequently confirmed by karyotype analysis of cultured amniotic fluid cells (AFC). As a result of recent developments in and clinical use of next-generation sequencing (NGS)-based noninvasive prenatal diagnosis (NIPD) technologies, an unprecedentedly large number of fetuses with 18T have been screened, and 18T has been identified as a leading cause of miscarriages (*3-5*).

Typically, 18T is characterized by significant growth delay, dolichocephaly, specific facies, limb anomalies, and visceral malformations (*2*). Chromosome 18 has the lowest gene density among human chromosomes (*6*). Unlike other trisomy syndromes such as trisomy 21, knowledge about the pathology and molecular mechanisms of 18T is very limited (*7,8*). This may be partly due to the lack of disease-associated samples and disease models as a result of the syndrome's rareness and extremely high mortality rate (*2,9*).

The recent development of induced somatic cell reprogramming efficiently provides induced pluripotent stem cells (iPSCs) from nearly all types of patient-derived somatic cells, allowing researchers to mimic pathological processes by differentiating iPSCs into disease-associated cells. This can provide novel clues to better understand the biological characteristics of many rare diseases such as 18T. A previous study attempted to establish 18T-iPSCs (*10*). Although

Released online in J-STAGE as advance publication May 15, 2018.

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pluripotent marker genes were expressed and exhibited pluripotency, the iPSC-like cells lost the extra chromosomes and converted to diploid cells after 10 passages.

To the extent known, the current study is the first to establish typical 18T-iPSCs that exhibited stable pluripotency and trisomy. These 18T-iPSCs exhibited expression profiles that differed significantly from those of normal AFC-derived iPSCs, suggesting new clues to understanding the mechanisms of 18T in embryo and disease development.

2. Materials and Methods

2.1. *AFC sampling and ethnical approval*

Ten-mL AFC samples were obtained from a pregnant woman who underwent an amniocentesis for prenatal diagnosis at Zibo Maternal and Child Health Hospital in Zibo, Shandong, China. Samples were obtained with the woman's informed consent. 18T was diagnosed after chromosomal microarray analysis. The study protocol has been reviewed and approved by the ethics committee of the Shandong Medical Biotechnological Center, Jinan, Shandong, China. After collection, AFC samples were centrifuged at 200 g for 5 minutes at room temperature, and then the cells were washed and cultured with BIOAMF-2 complete medium (Biological, Inc., Kibbutz Beit Haemek, Israel) at 37℃ in a 5% CO_2 atmosphere.

2.2. *iPSC reprogramming and culture*

iPSCs were generated using the procedure that was previously reported (*11-13*). Briefly, 2×10^6 AFC cells were transfected with the plasmids pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, pCXLE-hUL, and pCXWB-EBNA1 (kindly provided by Dr. Fabin Han of Liaocheng People's Hospital, Liaocheng, Shandong, China) through electrotransfection (Lonza Nucleofector and Nucleofector Kit V, Lonza, USA). On day 5 post-transduction, BIOAMF-2 culture medium was changed to iM1 (Osinglay, China). Medium was then replenished every day for 3 weeks, and medium was then changed to BioCISO (Osinglay, China) until uniform colonies were generated. The iPSC colonies were collected on day 26.

2.3. *Characterization of iPSCs using alkaline phosphatase staining, karyotyping, and detection of pluripotency markers*

Alkaline phosphatase staining was performed using the BCIP/NBT Kit (CoWin Biosciences, China) after treatment with 4% polyoxymethylene. Karyotyping of 18T-iPSCs was performed as described previously, and the Metafer karyotyping system (Carl Zeiss AG, Jena, Germany) was used to analyze images. The level of expression of the pluripotency biomarkers NANOG, SOX2, and OCT3/4 in 18T-iPSCs and derived AFCs was compared using real-time PCR. The primers used are listed in Table 1. After dissociation *via* 0.5-μM EDTA digestion and fixation, flow cytometry analysis was used to examine human pluripotency markers with the following antibodies: anti-Oct4 (BD Biosciences, USA), anti-SSEA4 (BD Biosciences, USA), anti-Tra-1-60 (BD Biosciences, USA), and anti-Tra-1-81 (BD Biosciences, USA). Flow cytometry was performed using the BD FACS Aria flow cytometer (BD Biosciences, USA).

2.4. *Embryoid body (EB) formation assay*

For an *in vitro* embryoid body (EB) formation assay, 18T-iPSCs were scraped from plates after dissociation using BioC-PDE1 (Osinglay, China) and cultured in 6-well suspension culture plates with BioCISO-EB1 medium (Osinglay, China) for 7 days to obtain EBs. The EBs were then cultured in 6-well culture plates coated with Matrigel for 7 to 14 days. Cells were then collected, and expression profiles of marker genes in the three germ layers were determined using real-time PCR. Corresponding genes and their primers are listed in Table 1.

2.5. *Teratoma formation assay*

For an *in vivo* teratoma formation, iPSCs were cultured to approximately 85% confluence. After 10-15 minutes of dissociation using Bio-PDE1, cells were scraped from the plates. One hundred and thirty μl of culture medium, 70 μL of Matrigel, and the cell suspension were subcutaneously injected into NOD/SCID mice. After 4-6 weeks, mice were sacrificed, and tumors that developed were fixed in formalin for 24 h and then embedded in paraffin. The specimens were stained with hematoxylin and eosin.

2.6. *Transcriptomic profiles according to RNA sequencing*

The global gene expression profiles of 18T-iPSCs and two normal iPSC lines established from ATCs by this Laboratory were analyzed using RNA-Seq technology. Briefly, total RNA was extracted using the TRIzol reagent (Invitrogen), a library was constructed, and RNA was sequenced with the Illumina HiseqX platform (illumine, USA). RNA sequencing data were expressed as fragments per kilobase of exon per million fragments mapped (FPKM). The Benjamini–Hochberg false discovery rate (FDR) threshold ≤ 0.05 and log2 fold change ≤ -1 or ≥ 1 based on DESeq-normalized read counts were used as criteria to determine differentially expressed genes.

Table 1. List of primers for pluripotency and differentiation markers

Target genes		Primer sequence
OCT4	Forward	CCTCACTTCACTGCACTGTA
	Reverse	CAGGTTTTCTTTCCCTAGCT
SOX2	Forward	CCCAGCAGACTTCACATGT
	Reverse	CCTCCCATTTCCCTCGTTTT
NANOG	Forward	AAGGTCCCGGTCAAGAAACAG
	Reverse	CTTCTGCGTCACACCATTGC
Actin	Forward	CCCAGAGCAAGAGAGG
	Reverse	GTCCAGACGCAGGATG
FOXA2	Forward	CCAACCCCACAAAATGGA
	Reverse	ATAATGGGCCGGGAGTACA
SOX17	Forward	ACCGCACGGAATTTGAAC
	Reverse	GCAGTAATATACCGCGGAGC
BRACHYURY	Forward	CCCTATGCTCATCGGAACA
	Reverse	TTCCAAGGCTGGACCAAT
MSX1	Forward	TCCGCAAACACAAGACGA
	Reverse	ACTGCTTCTGGCGGAACTT
MAP2	Forward	TGAAGCAAAGGCACCTCAC
	Reverse	TATGGGAATCCATTGGCG
PAX6	Forward	TTGCTTGGGAAATCCGAG
	Reverse	TGCCCGTTCAACATCCTT
GAPDH	Forward	GGTGGTCTCCTCTGACTTC
	Reverse	CTCTTCCTCTTGTGCTCTTG

3. Results

The induction of iPSCs from 18T-derived AFCs is summarized in Figures 1A. Figure 1B-1D shows the characteristic morphological changes during iPSC induction from AFCs. At 26-32 days of induction, the 18T-iPSCs were purified and exhibited typical phenotypes like embryonic stem (ES) cells (Figure 1D).

After 18 passages, the 18T-iPSCs exhibited stable 18T karyotypes (Figure 1E). Alkaline phosphatase-positive ES cell-like colonies of 18T-iPSCs were noted (Figure 1F), and real-time PCR and flow cytometry staining revealed significant expression of pluripotent markers (Figures 1F-G). In addition, the generated 18T-iPSCs were found to have differentiated into all three germ layers according to the *in vitro* EB formation assay and the *in vivo* teratoma formation assay (Figure 2D). Taken together, these findings indicate that the established 18T-iPS cell line has obvious pluripotency while maintaining trisomy.

RNA-seq was used to analyze the transcriptomic profile of the 18T-iPSCs in comparison to that of normal AFC-derived iPSCs, and a list of significantly differentially expressed genes was identified (Table 2, Figure 3). Interestingly, a series of genes associated with organ differentiation (the brain, testis cryptorchidism, heart, skin, kidneys, esophagus, bone, and lungs) was found to be differently expressed in 18T-iPSCs compared to normal iPSCs, and these differences in expression may partly account for the comprehensive malformations caused by 18T.

4. Discussion

The current study successfully generated an iPSC line from 18T AFCs. Findings indicated that these 18T-iPSCs were alkaline phosphatase-positive and significantly expressed pluripotency biomarkers such as NANOG, SOX2, OCT3/4, and SSEA4. As further corroboration, these 18T-iPSCs have the potential to differentiate into three germ layers both *in vitro* and *in vivo*. Unlike the 18T-iPS cell line established by Li *et al*. (*10*), the current cell line exhibited stable trisomy after numerous passages. Therefore, the 18T-iPSCs generated in the current study are more representative of 18T.

The current study also observed the genomic instability of 18T-iPSCs in another iPSC line derived from the same AFCs with 18T pregnancies. Although those cells exhibited pluripotency, they spontaneously gained an extra chromosome 8 after more than 10 passages. The current findings confirmed the contention that 18T cells are prone to spontaneously differentiating, but they have built upon results of previous studies by revealing an extra trisomy mutation. Therefore, whether the 18T-iPSCs established here would exhibit genomic instability after more passages is a question that warrants study.

18T is an aberrantly differentiated condition with complicated malformations, and the exact molecular mechanism by which an extra 18 chromosome causes that pathology is not clear (*10*). Interestingly, the current transcriptomic findings indicated that the gene expression of 18T-iPSCs varied significantly from that in normal iPSCs, but the genes in question were not limited to those located on chromosome 18. Moreover, differentiation-associated genes were markedly dysregulated in 18T-iPSCs, suggesting that 18T may involve an aberrant genetic background in the ES cell stage.

In conclusion, this study successfully established a representative 18T-iPSC clone, and it described the molecular characteristics of that clone at the transcriptomic level for the first time. These findings should provide more information with which to understand the pathology of 18T. This 18T-iPSC clone also provides a useful tool with which to study aberrant multi-lineage differentiation in 18T.

Acknowledgements

This work was supported by a grant from the Innovation Project of the Shandong Academy of Medical Science.

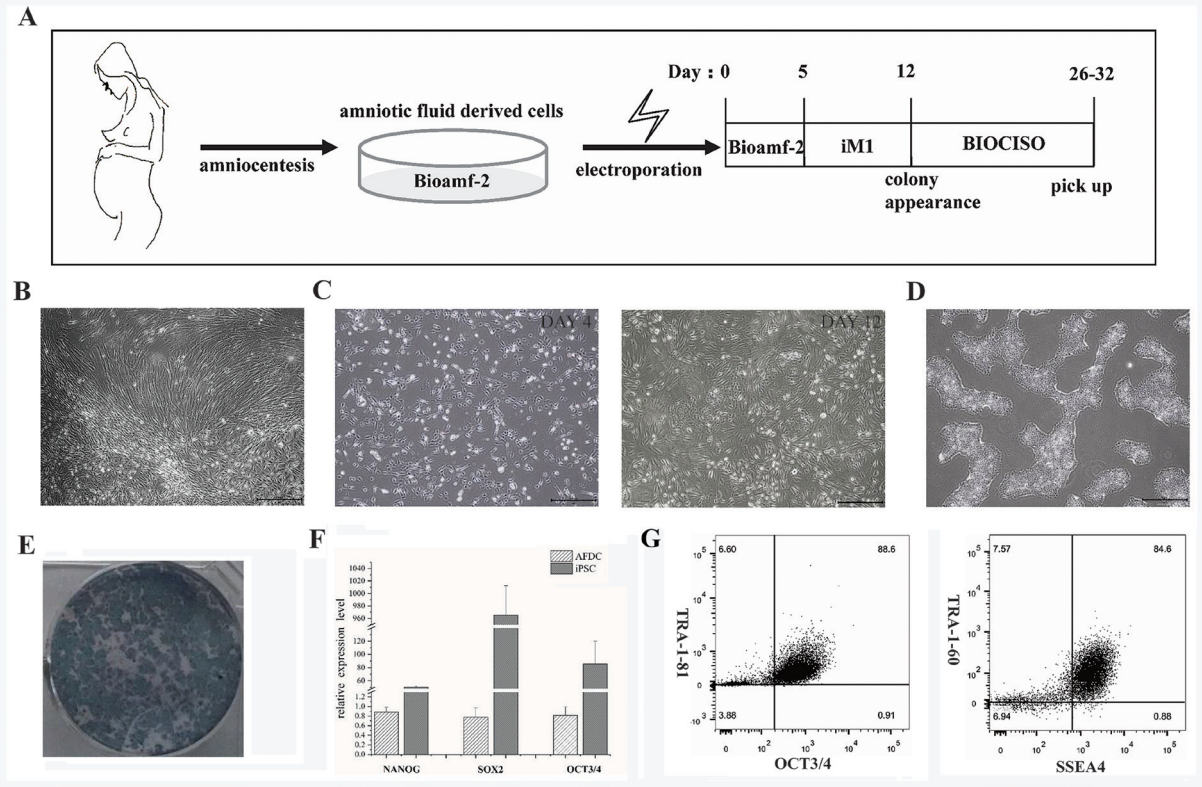

Figure 1. Induction and characterization of 18T-iPSCs from AFC. (A) Timing of iPSCs induction; **(B)** representative images of cells from amniotic fluid cells; **(C)** Day 4 and 12; **(D)** Purified 18T-iPSCs (Scale bar = 500 μm); **(E)** alkaline phosphatase staining; **(F)** Pluripotency biomarkers of 18T-iPSCs detected with real-time PCR and **(G)** flow cytometry.

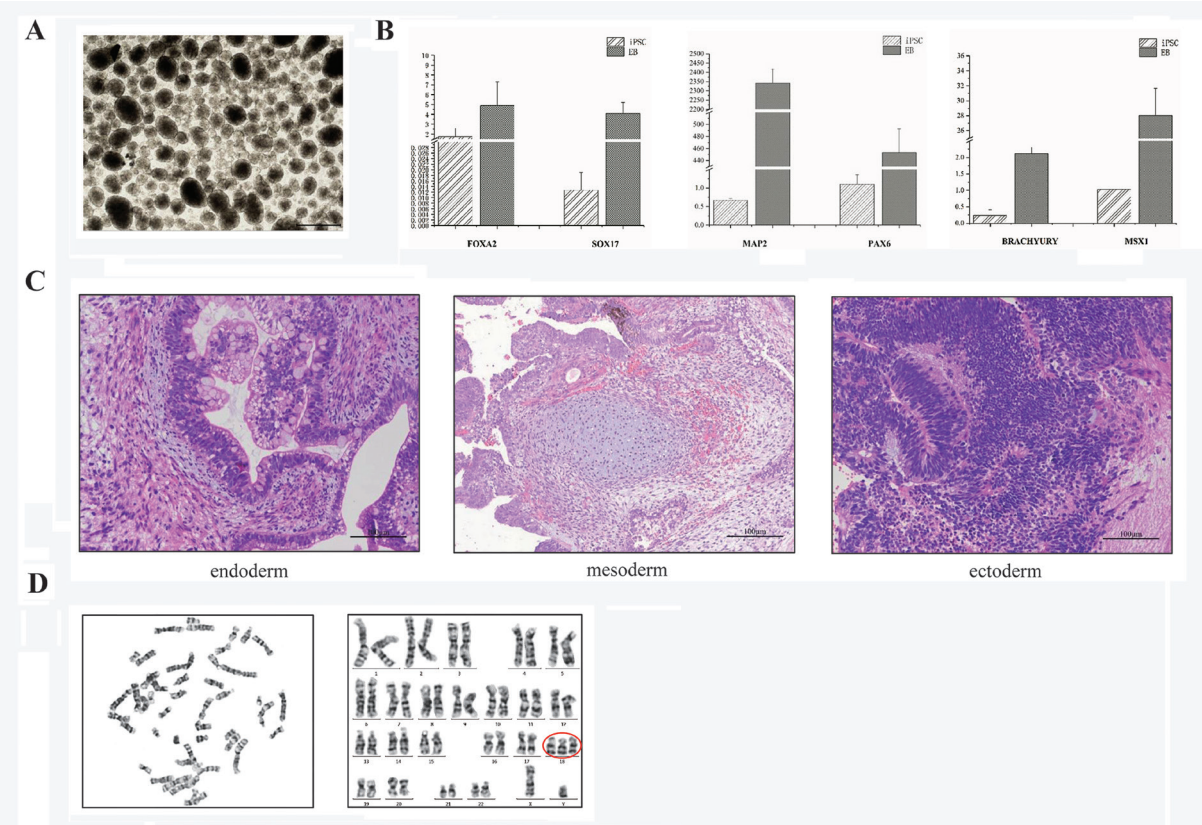

Figure 2. Confirmation of the differentiation potential and trisomy of 18T-iPSC. (A) Embryoid body (EB) formation by 18T-iPSCs (Scale bar = 500 μm); **(B)** Detection of EB biomarkers with real-time PCR; **(C)** Hematoxylin and eosin staining of teratoma derived from a 18T-iPSC clone (Scale bar = 100 μm); **(D)** Karyotype analysis in G-band staining of 18T-iPSCs; chromosome 18 is circled in red.

Table 2. List of significantly differentially expressed genes

Gene ID	Gene symbol	Regulation and expression	
ENSG00000087250	MT3	down	Biased expression in the brain (RPKM 149.3) and adrenal glands (RPKM 10.1)
ENSG00000111262	KCNA1	down	Biased expression in the brain (RPKM 6.1) and thyroid (RPKM 0.3)
ENSG00000151952	TMEM132D	down	Biased expression in the brain (RPKM 5.0) and testes (RPKM 0.4)
ENSG00000105376	ICAM5	down	Biased expression in the brain (RPKM 11.7), lungs (RPKM 3.3), and 1 other type of tissue
ENSG00000196990	FAM163B	down	Biased expression in the brain (RPKM 9.7) and adrenal glands (RPKM 1.9)
ENSG00000143847	PPFIA4	down	Biased expression in the brain (RPKM 8.9), heart (RPKM 8.4), and 10 other types of tissue
ENSG00000136928	GABBR2	down	Restricted expression in the brain (RPKM 33.5)
ENSG00000183775	KCTD16	down	Biased expression in the brain (RPKM 4.6), lungs (RPKM 0.4), and 3 other types of tissue
ENSG00000129990	SYT5	down	Biased expression in the brain (RPKM 20.3) and adrenal glands (RPKM 2.1)
ENSG00000125462	C1orf61	down	Restricted expression in the brain (RPKM 43.3)
ENSG00000141837	CACNA1A	down	Biased expression in the brain (RPKM 5.1), stomach (RPKM 1.8), and 6 other types of tissue
ENSG00000161082	CELF5	down	Biased expression in the brain (RPKM 14.1), ovaries (RPKM 2.1), and 1 other type of tissue
ENSG00000181418	DDN	down	Biased expression in the brain (RPKM 36.7) and kidneys (RPKM 8.9)
ENSG00000165973	NELL1	down	Biased expression in the brain (RPKM 7.1), kidneys (RPKM 6.6), and 2 other types of tissue
ENSG00000138650	PCDH10	down	Biased expression in the brain (RPKM 21.3), placenta (RPKM 10.2), and 4 other types of tissue
ENSG00000242173	ARHGDIG	down	Biased expression in the brain (RPKM 34.7), duodenum (RPKM 7.8), and 4 other types of tissue
ENSG00000177181	RIMKLA	down	Biased expression in the brain (RPKM 2.6), colon (RPKM 1.6), and 10 other types of tissue
ENSG00000131094	C1QL1	down	Biased expression in the brain (RPKM 10.8), kidneys (RPKM 1.7), and 4 other types of tissue
ENSG00000133019	CHRM3	down	Biased expression in the brain (RPKM 5.0), salivary glands (RPKM 2.4), and 12 other types of tissue
ENSG00000111674	ENO2	down	Biased expression in the brain (RPKM 167.6), adrenal glands (RPKM 26.3), and 9 other types of tissue
ENSG00000105605	CACNG7	down	Restricted expression in the brain (RPKM 18.0)
ENSG00000166342	NETO1	up	Biased expression in the brain (RPKM 5.4) and spleen (RPKM 0.5)
ENSG00000160471	COX6B2	down	Restricted expression in the testes (RPKM 26.9)
ENSG00000151948	GLT1D1	down	Biased expression in the testes (RPKM 6.9), bone marrow (RPKM 6.4), and 9 other types of tissue
ENSG00000175513	TSGA10IP	down	Restricted expression in the testes (RPKM 2.7
ENSG00000204961	PCDHA9	down	Biased expression in the testes (RPKM 14.6), endometrium (RPKM 1.3), and 2 other types of tissue
ENSG00000130270	ATP8B3	down	Biased expression in the testes (RPKM 14.6), endometrium (RPKM 1.3), and 2 other types of tissue
ENSG00000205129	C4orf47	down	Biased expression in the testes (RPKM 3.0), thyroid (RPKM 0.8), and 5 other types of tissue
ENSG00000162039	MEIOB	down	Restricted expression in the testes (RPKM 20.3)
ENSG00000248746	ACTN3	down	Biased expression in the testes (RPKM 1.6), prostate (RPKM 0.6), and 2 other types of tissue
ENSG00000118407	FILIP1	down	Biased expression in the heart (RPKM 16.2), esophagus (RPKM 4.3), and 12 other types of tissue
ENSG00000106631	MYL7	up	Restricted expression in the heart
ENSG00000130226	DPP6	up	Biased expression in the brain (RPKM 22.2), endometrium (RPKM 11.9), and 5 other types of tissue
ENSG00000169035	KLK7	down	Biased expression in the skin (RPKM 68.1) and esophagus (RPKM 25.3); Polymorphisms in this gene may play a role in the development of atopic dermatitis.
ENSG00000182580	EPHB3	down	Broad expression in the skin (RPKM 18.6), stomach (RPKM 10.2), and 15 other types of tissue
ENSG00000129455	KLK8	down	Biased expression in the skin (RPKM 32.0) and esophagus (RPKM 29.8)
ENSG00000143590	EFNA3	down	Biased expression in the skin (RPKM 20.5), esophagus (RPKM 8.1), and 3 other types of tissue
ENSG00000183479	TREX2	up	Biased expression in the skin (RPKM 5.9), spleen (RPKM 0.7), and 5 other types of tissue
ENSG00000164879	CA3	down	Biased expression in the kidneys (RPKM 2.5), brain (RPKM 1.7), and 10 other types of tissue
ENSG00000119715	ESRRB	down	Biased expression in the kidneys (RPKM 4.2), heart (RPKM 1.8), and 2 other types of tissue
ENSG00000095932	SMIM24	down	Biased expression in the kidneys (RPKM 301.3), duodenum (RPKM 292.3), and 4 other types of tissue
ENSG00000161270	NPHS1	down	Biased expression in the kidneys (RPKM 12.8), pancreas (RPKM 3.0), and 1 other type of tissue
ENSG00000167755	KLK6	down	Biased expression in the esophagus (RPKM 23.8), brain (RPKM 23.7), and 4 other types of tissue
ENSG00000135046	ANXA1	up	Biased expression in the esophagus (RPKM 2850.3), bone marrow (RPKM 574.5), and 4 other types of tissue
ENSG00000206073	SERPINB4	up	Biased expression in the esophagus (RPKM 63.1), bladder (RPKM 11.6), and 1 other type of tissue
ENSG00000057149	SERPINB3	up	Restricted expression in the esophagus (RPKM 424.4)
ENSG00000012779	ALOX5	down	Broad expression in bone marrow (RPKM 35.1), the lungs (RPKM 26.8), and 15 other types of tissue
ENSG00000161835	GRASP	down	Broad expression in bone marrow (RPKM 8.3), fat (RPKM 7.8), and 23 other types of tissue
ENSG00000059804	SLC2A3	down	Biased expression in bone marrow (RPKM 183.0), the placenta (RPKM 54.5), and 11 other types of tissue
ENSG00000066926	FECH	up	Broad expression in bone marrow (RPKM 19.5), the kidneys (RPKM 8.4), and 24 other types of tissue
ENSG00000171848	RRM2	up	Biased expression in bone marrow (RPKM 28.1), lymph nodes (RPKM 20.5), and 13 other type of tissue
ENSG00000170561	IRX2	up	Biased expression in the lungs (RPKM 4.6), skin (RPKM 2.8), and 9 other types of tissue
ENSG00000105371	ICAM4	down	Biased expression in the lungs (RPKM 3.3), bone marrow (RPKM 3.1), and 13 other types of tissue
ENSG00000179344	HLA-DQB1	down	Broad expression in the lungs (RPKM 158.2), lymph nodes (RPKM 135.4), and 19 other types of tissue
ENSG00000090339	ICAM1	down	Broad expression in the lungs (RPKM 83.1), bone marrow (RPKM 40.5), and 20 other types of tissue
ENSG00000095383	TBC1D2	down	Broad expression in the lungs (RPKM 7.7), bladder (RPKM 3.8), and 23 other types of tissue

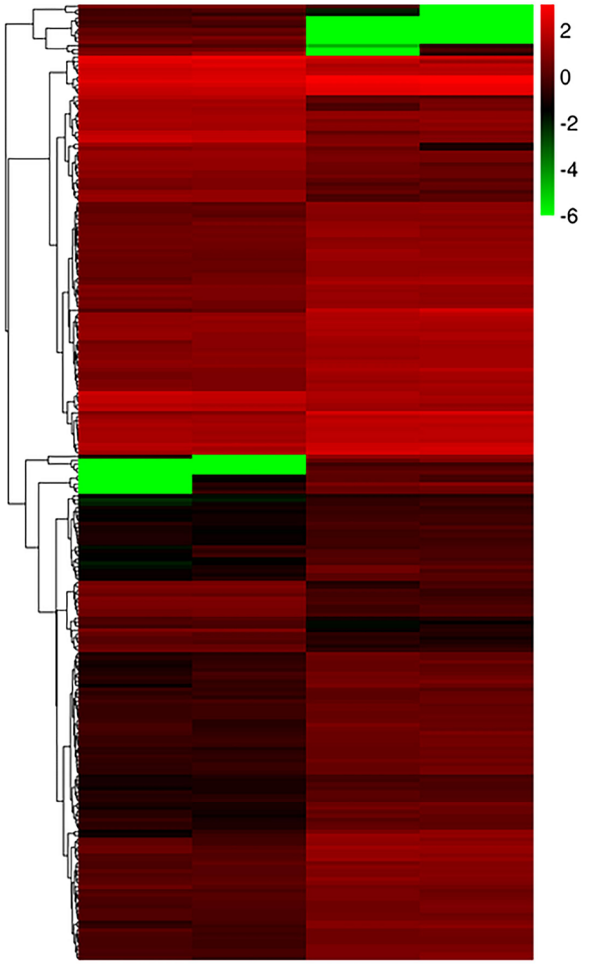

Figure 3. Hierarchical clustering analysis of the differentially expressed genes in 18T-iPSCs and normal karyotype iPSCs. Red indicates upregulated genes and green indicates downregulated genes.

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(Received April 16, 2018; Revised May 11, 2018; Accepted May 12, 2018)

Original Article

DOI: 10.5582/irdr.2018.01042

Possible predictive factors for recovery of left ventricular systolic function in Takotsubo cardiomyopathy

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Summary **Takotsubo cardiomyopathy (TTC) is a transient systolic dysfunction of the left ventricle which is usually seen in elderly women, often following a physical or emotional stressful event. Little is known about the prognostic factors affecting the recovery of systolic function. Thirty-six patients diagnosed with TTC from January 2006 to January 2017 at our hospital were included. Median time to recovery of ejection fraction (EF) was calculated to be 25 days. Early recovery of ejection fraction was defined as less than or equal to 25 days (group 1) and late recovery was defined as more than 25 days (group 2). Demographic and clinical factors were compared between the groups. Fifty percent patients had early recovery of EF with a mean time to recovery of 7.11 days and 50% had late recovery of ejection fraction with a mean time to recovery of 58.38 days. Younger age at presentation was associated with early recovery of systolic function (58.83 ± 2.7 years *vs*. 67.33 ± 2.7 years, $p = 0.032$). Presence of an identifiable triggering event was associated with early recovery (83% in group 1 *vs*. 50% in group 2, $p = 0.034$). Generalized anxiety disorder was seen more commonly in the group with early recovery (78% in group 1 *vs*. 45% in group 2, $p = 0.040$). In conclusion, younger age, generalized anxiety disorder and presence of triggering event were seen more commonly in patients with early recovery of left ventricular systolic function in Takotsubo cardiomyopathy.**

Keywords: Takotsubo, cardiomyopathy, left ventricular ejection fraction, predictors, recovery

1. Introduction

Takotsubo cardiomyopathy (TTC) or acute stress induced cardiomyopathy initially described in Japan in 1990, is increasingly being recognized around the globe with the characteristic features of apical systolic ballooning and absence of coronary obstruction while mimicking acute coronary syndrome (ACS) (*1*). The incidence of TTC has been reported to be 1.7% to 2.2% among patients presenting as ACS (*2*). Although there is no significant racial predilection, most of these reported cases were among Asian and Caucasian women. About 89% of the patients affected were female with an average age of 67.7 years (*3*). The pathogenesis involves myocardial stunning or direct toxicity by catecholamine surge associated with an emotional or physical stress (*4*). Clinical presentation is similar to ACS with chest pain, syncope, dyspnea, or symptoms of heart failure or as arrhythmias. ST and T changes are common on EKG and cardiac biomarkers are often elevated (*5*). Cardiac catheterization shows no significant coronary occlusion, but demonstrate the characteristic left ventricular apical ballooning during systole. The most commonly used diagnostic tool is the Mayo Clinic diagnostic criteria which requires all four criteria to be satisfied for the diagnosis of TTC (*6*).

In a position statement by Lyon *et al.*, recovery of left ventricular systolic dysfunction was attained by 12 weeks (*7*). However, another study by Park *et al*., on 26 patients with TTC showed that 20 of them had normalization of left ventricular function in 7 days making it impossible to define a clear-cut recovery

Released online in J-STAGE as advance publication May 23, 2018.

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period (*8*). 21.8% of the patients with TTC developed serious in-hospital complications which was similar to the rate in acute coronary syndrome patients (*5*). Multiple studies to identify the factors associated with poor survival and increased mortality have been published (*9-11*). Some of the factors identified were low left ventricular ejection fraction (LVEF), severe mitral regurgitation, increased pulmonary artery pressure and right ventricular involvement. The factors which affect time to recovery of left ventricular systolic function, however has not been widely reported (*12*). We aimed to identify these factors affecting recovery to generate a better understanding about the pathophysiology and try to fill the gap in knowledge regarding management of TTC. Since majority of the care and follow up until full recovery of TTC are done in outpatient settings following hospital discharge and has not been studied well, we aimed to include hospitalization data as well as from the outpatient follow up visits.

2. Materials and Methods

We conducted a retrospective review of the medical records of patients diagnosed with TTC at Monmouth Medical Centre from January 2006 till January 2017. The study was approved by the Institutional Review Board at our hospital. We identified patients diagnosed with TTC using the ICD 9/ICD 10 diagnostic codes. Electronic medical records (including scanned documents from previous paper charts) of these patients were reviewed. We included patients who were more than 18 years old, had transient akinesia or dyskinesia of left ventricular (LV) apical and mid-ventricular segments with regional wall motion abnormalities extending beyond a single epicardial vascular distribution and new EKG abnormalities (either ST segment elevation and/or T wave inversion) or modest elevation in cardiac troponins as per the Revised Mayo Clinic Diagnostic Criteria (*6*). The exclusion criteria were presence of obstructive coronary disease or angiographic evidence of acute plaque rupture, myocarditis or pheochromocytoma. From a total of 45 patients with diagnosis of TTC during the study period, 36 patients were included for analysis. 7 patients were excluded due to lack of follow up echocardiograms and 2 patients were excluded due to lack of coronary angiography.

The echocardiograms of patients who met the inclusion criteria at the time of diagnosis and follow up were reviewed by an independent board certified cardiologist and LVEF was determined using Modified Simpson Rule (*13*). Follow up echocardiograms were obtained for all patients at these time intervals: 1 week, 4 to 6 weeks, 12 weeks, 6 months, 9 months and 1 year after the event and the data was obtained from the outpatient cardiology records. A value of LVEF more than or equal to 50% on follow-up was defined as recovery.

Based upon one-year follow up data, we calculated 25 days to be the sample median for recovery time of LVEF. Patients were then divided into 2 groups; group 1 included patients with early recovery before or at 25 days and group 2 included patients with delayed recovery after 25 days of the event. We used median as a measure of central tendency because of the presence of an outlier, one of the patients had a recovery of left ventricular systolic function at 12 months while rest of the patients recovered in ≤ 12 weeks. Figure 1 summarizes the study in a flowchart. Demographic and clinical characteristics were compared. Presence of generalized anxiety disorder was considered positive if the patient was taking prescription anxiolytic agents with a diagnosis of anxiety disorder made by a psychiatrist as an out-patient. A triggering event was defined as a stressful incident immediately before admission that resulted in significant physiological disturbance capable of having acute cardio-vascular affect. Triggering events were further categorized as emotional or physical. Emotional triggers included those causing psychosocial stress such as grief, anger, anxiety, fear. Physical stress included exacerbation of chronic medical conditions, medical or surgical procedures or any acute illness. Cardiogenic shock, cardiac arrest, life threatening arrhythmias, congestive heart failure and death were considered as adverse cardiac events.

Categorical variables were expressed as numbers or percentages and continuous variables were expressed as mean ± standard deviation or median with first inter-quartile range. Continuous variables were compared between the two groups using Student *t* test or Mann-Whitney *U*-test. Categorical variables were compared using χ^2 or Fischer exact test. Statistical significance was defined as a p value < 0.05. All data were analyzed using SPSS 20.0 (SPSS Inc Chicago, Illinois)

3. Results

The study population included 36 patients with a mean age of 63.08 ± 12.06 years. Majority of the population (83.3%) were women. The prevalence of psychiatric disorders in the patient population was 61.11%, diabetes mellitus 13.89% and hypertension 47.22%. A triggering event preceding presentation was documented in 66.67%. Physical stressors which included surgical procedures, and acute non-cardiac illness like hypoxic respiratory failure from community acquired pneumonia, ischemic stroke, COPD exacerbation, alcohol intoxication and small bowel obstruction were more common (50%). Emotional stressors such as extreme grief, anger, anxiety and fear were documented in 16.67% of the patients. The baseline clinical and demographic characteristics of all

patients is summarized in Table 1.

The mean recovery period was calculated to be 32.2 days (median; 25 days, SD 19.95). Presence of a single outlier value resulted in this difference in mean and median. 18 patients (50%) had recovery of LVEF in 25 days or less (Group 1) and 18 patients (50%) patients had recovery of function after 25 days. Mean time to recovery was 7.1 days in Group 1 and 58.4 days in Group 2. There was statistically significant difference in age at presentation between Group 1 and Group 2 (58.83 ± 2.7 *vs*. 67.33 ± 2.7 years, $p = 0.032$). Gender distribution was not statistically different in both the groups. Prevalence of generalized anxiety disorder was significantly higher in group 1 (77.78% *vs*. 44.44%, $p = 0.040$). Group 1 also had more documented preceding physical or emotional trigger (83.33% *vs*. 50%, $p = 0.034$). Prevalence of other comorbid conditions like diabetes mellitus, hypertension, autoimmune disorders, and hyperlipidemia and tobacco dependence did not differ significantly between the two groups. Cardiac enzyme levels, BNP levels and EKG changes were not significantly different between the two groups. Comparison of demographic and clinical characteristics of the two groups is shown in Table 2.

Clinical Course and treatment (Table 3): Left ventricular systolic dysfunction on presentation was not significantly different between the two groups (32.11 ± 8.32% *vs*. 36.34 ± 10.49%, $p = 0.188$). Majority of the population had apical form of TTC in both groups. Among patients with early recovery of systolic function, 11.11% of patients were on angiotensin converting enzyme inhibitors or angiotensin receptor blockers prior to presentation and 11.11% were using

Table 1. Baseline Characteristics of total population

Variable	Incidence ($n = 36$)
Age (years, Mean ± SD)	63.1 ± 12.1
Female Sex (%)	30 (83.3%)
Baseline EF (%, Mean ± SD)	62.0 ± 5.3
Mean Acute EF (%, Mean ± SD)	34.2 ± 9.6
Alcohol dependence (%)	3 (8.3%)
Tobacco dependence (%)	16 (44.4%)
Co-morbid medical conditions	
Hypertension	17 (47.2%)
Diabetes Mellitus	5 (13.9%)
Cancer	8 (22.2%)
Psychiatric disorder	22 (61.1%)
Neurological disorder	3 (8.3%)
COPD	8 (22.2%)
Autoimmune disorder	4 (11.1%)
Triggering event	66.7%
Physical trigger	50%
Emotional trigger	16.7%

Table 2. Demographic and clinical characteristics of the two groups

Variable	Early recovery of EF (≤ 25 days), $n = 18$	Late recovery of EF (> 25 days), $n = 18$	p-value
Age – years (Mean ± SD)	58.8 ± 2.7	67.3 ± 2.7	0.032
Female Sex	14 (77.8%)	16 (88.9%)	0.371
Triggering event	15 (83.3%)	9 (50%)	0.034
Co-morbid medical condition			
Cancer	4 (22.2%)	4 (22.2%)	1.000
Hypertension	6 (33.3%)	11 (61.1%)	0.096
Diabetes	2 (11%)	3 (16.7%)	0.232
Generalized anxiety	14 (77.8%)	8 (44.4%)	0.040
Neurological disorder	2 (11.1%)	1 (5.6%)	0.363
COPD	6 (33.3%)	2 (11.1%)	0.108
Autoimmune disorder	0	4 (22.2%)	0.104
HIV	1 (5.6%)	0	1.000
Alcohol dependence	2 (11%)	1 (5.6%)	0.288
Tobacco dependence	10 (55.6%)	6 (33.3%)	0.179
Medications prior to admission			
ACE inhibitor	2 (11.1%)	3 (16.7%)	0.630
Beta blocker (non-selective, selective)	2 (11.1%)	6 (33.3%)	0.109
Dihydropyridine calcium channel blocker	2 (11.1%)	1 (5.6%)	0.547
Diuretic (Loop & Thiazide)	2 (11.1%)	2 (11.1%)	0.699
Anti-platelets	5 (27.8%)	6 (33.3%)	0.840
Statins	3 (16.7%)	2 (11.1%)	0.630
Presenting EF (Mean ± SD)	32.1 ± 8.3	36.3 ± 10.5	0.188
EKG changes (%)			
ST segment elevation	8 (44.4%)	4 (22.2%)	0.158
T wave inversion	7 (38.9%)	8 (44.4%)	0.729
Peak Troponins (ng/L) (Mean ± SD)	3.3 ± 3.7	3.3 ± 5.3	0.435
BNP level (pg/mL) (Mean ± SD)	655.14 ± 769.0	584.98 ± 501.97	0.826
Lipid profile (Mean ± SD)			
Total Cholesterol (mg/dL)	168.8 ± 46.9	165.1 ± 32.7	0.817
HDL (mg/dL)	55.3 ± 23.5	51.8 ± 10.9	0.627
LDL (mg/dL)	96.7 ± 42.1	92.4 ± 30.8	0.761
Triglycerides (mg/dL)	99.4 ± 41.5	116 ± 73.1	0.458

Table 3. Features on presentation and medication initiated

Variable	Early recovery of EF (≤ 25 days), *n* = 18	Late recovery of EF (> 25 days), *n* = 18	*p*-value
Echocardiographic morphology			
Apical	17 (94.4%)	16 (88.9%)	1.000
Mid-ventricular	0	1 (5.6%)	1.000
LVOT[†]	1 (5.6%)	1 (5.6%)	1.000
RV[‡] apical akinesia	0	1 (5.6%)	1.000
Inverted	1 (5.6%)	0	1.000
Recurrence of TTC	1 (5.6%)	0	1.000
> 50% Coronary artery occlusion	2 (11.1%)	1 (5.6%)	0.546
Medications initiated			
Beta blocker	13 (72.2%)	13 (72.2%)	1.000
Anti-platelets	9 (50%)	4 (22.2%)	0.083
ACE inhibitor	12 (66.7%)	11 (61.1%)	0.786
Diuretics	2 (11.1%)	3 (16.7%)	0.630
Statins	4 (22.2%)	2 (11.1%)	0.371
Digoxin	1 (5.6%)	2 (11.1%)	0.546
EF on recovery	60.1 ± 5.2	57.2 ± 7.3	0.180
Change in EF	28.1 ± 10.7	20.1 ± 13.1	0.054

[†] Left ventricular outflow tract obstruction. [‡] Right ventricle.

beta blockers; the utilization increased to 66.67% and 72.22% respectively on discharge. Similarly, in group 2, 16.67% were using angiotensin converting enzyme inhibitors or angiotensin receptor blockers and 33.33% were using beta blockers, the utilization increased to 61.11% and 72.22% respectively at discharge. There was no statistically significant difference identified regarding medication usage between the two groups. The recovery EF was similar in both groups, 60.13 ± 5.23% in group 1 and 57.23 ± 7.31% in group 2 (p = 0.180). The early recovery group had an average EF improvement of 28.1% while late recovery group had only 20.1% on up to one year follow up.

No significant difference was found in the incidence of cardiogenic shock (1 patient in Group 2), arrhythmias (2 patients in Group 2), cardiac arrest (1 in group 1 *vs*. 2 in Group 2) and chronic congestive heart failure (1 patient in Group 1 *vs*. 3 in Group 2) between the two groups. One patient in the late recovery group died of cardiac arrest within a year. The overall number of patients with adverse outcomes were significantly higher in the late recovery group (50% *vs*. 11.11%, p = 0.011).

4. Discussion

We found that the group with early recovery was almost a decade younger than the group with delayed recovery. In a study by Citro *et al*. on TTC in older adults it was found that in-hospital complication rate for adults aged 75 or older was higher than those younger than 75 (*14*). This study shed light on the impact of age on TTC. A senescent heart has reduced capacity to recover from stress as fibroblasts have a diminished response to stimulatory signals, which may explain why it would take older adults longer to recover the left ventricular systolic function in TTC (*15,16*).

Presence of a triggering event preceding the onset of TTC was also associated with early recovery. Physical stressors seen in our patient population included hypoxic respiratory failure requiring intubation due to sepsis from community acquired pneumonia, small bowel obstruction managed medically, ischemic stroke with resolution of symptoms with tissue plasminogen activator, COPD exacerbation, alcohol intoxication and post endoscopic sinus surgery. Many of these physical stressful events were managed successfully with resolution of symptoms. Emotional triggers included anticipation of major surgery, loss of parent and argument with neighbor. We hypothesize that the presence of a stressful event, physical or emotional, once identified, if managed appropriately can lead to early recovery of TTC. In the absence of a known triggering event we are left with empirical management of TTC, while the cause may persist. It has been studied previously that in-hospital death is lower in patients where TTC was triggered by emotional stress or those not associated with a stressful trigger (*17*). In another study, being male and having a physical trigger were independent risk factors for in-hospital mortality (*18*). However no study has been done to investigate the effect of triggering event on the recovery of left ventricular systolic function. One must be aware that severity of the triggering event can have an impact on recovery, majority of the patient population in our study had triggering events that could be managed successfully with complete resolution.

Prevalence of generalized anxiety disorder was significantly higher in the group with early recovery. Anxiety has been seen more frequently in patients with TTC (*19*). If a patient has a known history of anxiety it is more likely to be noticed and managed appropriately while it may be overlooked in a patient with no known history. There may be a component of anxiety in many

cases of TTC which if addressed adequately with medications or psychiatric intervention may aid in early recovery. Detailed chart analysis of our cohort showed that patients with history of anxiety disorder were also treated more often with benzodiazepines such as lorazepam and alprazolam as compared to those with no known history of anxiety disorder; 86.4% of those with known history of anxiety disorder *vs*. 28.6% with no known history of anxiety disorder. Further studies are needed to look into the application of benzodiazepines as anxiolytics in the management of acute phase of TTC.

We acknowledge the limitations of our study. It must be noted that the sample size of our study was small and it was a single center study, hence one must be careful before extrapolating the data to a larger population. Secondly, ours was a retrospective study thus subject to missing or inaccurate reporting of events. One cannot rule out previous subclinical episodes of TTC in these patients especially the cohort with delayed recovery of ejection fraction. The clinical implications of our study should be considered as a hypothesis for prospective investigation in a larger cohort.

Nonetheless, we recommend a thorough search for a triggering event in patients with TTC, as managing the cause may lead to early recovery. We suggest that interventions to alleviate anxiety levels in patients with known history of anxiety should be undertaken, while keeping a low threshold for treating anxiety in patients with no known history.

5. Conclusion

According to our study, younger age, presence of a clear triggering event and generalized anxiety may be associated with early recovery of left ventricular systolic function in TTC. Delayed recovery is associated with a clinical course complicated with cardiac arrest, arrhythmias, cardiogenic shock and congestive heart failure. Further large-scale studies need to be done to identify factors predicting early recovery which will help in formulating management guidelines that are essentially absent at present.

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(Received April 30, 2018; Revised May 15, 2018; Accepted May 16, 2018)

Brief Report

DOI: 10.5582/irdr.2018.01047

The expression and clinicopathological role of CDX2 in intrahepatic cholangiocarcinoma

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Summary **The aim of this study was to examine the expression and clinicopathological role of caudal homeobox 2 (CDX2) in intrahepatic cholangiocarcinoma (ICC). CDX2 expression was determined immunohistochemically in 93 patients with ICC. The association between CDX2 expression and clinicopathological features of ICC was also examined in patients with ICC. Immunohistochemical staining for CDX2 was noted in 27 patients (29.03%); patients with CDX2-positive tumors had significant survival advantages over those with CDX2-negative tumors (median survival was 40 months for patients with CDX2-positive tumors and 13 months for patients with CDX2-negative tumors; the hazard ratio was 0.36, the 95% confidence interval was 0.22-0.59, and $p < 0.001$). The rate of CDX2 expression was 13.46% in patients with lymphatic invasion and 48.78% in patients without lymphatic invasion ($\chi^2 = 13.88$, $p < 0.01$); positivity for CDX2 expression was significantly higher in patients with well-differentiated or moderately differentiated tumors than that in patients with poorly differentiated tumors (41.7% in patients with well-differentiated tumors, 47.6% in patients with moderately differentiated tumors, and 20.0% in patients with poorly differentiated tumors; Mann-Whitney *U* test, $p = 0.01$). In addition, CDX2 expression differed significantly in patients with ICC due to hepatolithiasis and patients with ICC not due to hepatolithiasis (36.51% and 13.33%, respectively, $\chi^2 = 5.30$, $p = 0.02$). Positivity for CDX2 expression resulted in significant survival advantages for patients with ICC. CDX2 might be used as a prognostic marker in patients with ICC.**

Keywords: Caudal homeobox 2 (CDX2), intrahepatic cholangiocarcinoma, immunohistochemistry, hepatolithiasis, clinicopathological features, prognosis

1. Introduction

Intrahepatic cholangiocarcinoma (ICC) accounts for approximately 10% to 20% of primary liver cancers, second only to hepatocellular carcinoma (*1-5*). Depending on its anatomical location, ICC is classified into one of three categories of cholangiocarcinoma (intrahepatic, hilar, or extrahepatic). Histologically, ICC is considered to be the least common of the three categories (*4*). The incidence and mortality of ICC have been increasing worldwide over the past 30 years, while the incidence of all other forms of cholangiocarcinoma has been declining slightly (*5-11*). Thus far, several risk factors for ICC have been identified, including liver fluke infection, primary sclerosing cholangitis, and hepatolithiasis; hepatolithiasis has been found to be etiologically related to the development of ICC (*12-17*). Surgery is the main form of treatment and it offers the hope of prolonged survival for patients with ICC. However, postoperative long-term outcomes are poor, with a 5-year survival rate of around 30% to 35% (*18*). Similarly, locoregional neoadjuvant or palliative therapies have not been found to offer any significant survival advantages (*19-22*).

Advances in tumor biology have greatly facilitated the use of bio-molecular markers from biopsy, serum,

Released online in J-STAGE as advance publication May 25, 2018.

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or postoperative specimens to predict tumor behavior. Therefore, novel prognostic predictors for early diagnosis, prevention, and treatment of ICC need to be identified.

A member of the caudal-related homeobox gene family, caudal homeobox 2 (CDX2) is commonly expressed in small and large epithelial cells (*23*). CDX2 has been found to play important roles in the proliferation and differentiation of intestinal epithelial cells (*23,24*). Ectopic CDX2 expression is regarded as a sensitive marker indicating intestinal metaplasia in Barrett's esophagus and the stomach, and this finding has been corroborated both in vivo and in vitro (*25-29*). The prognostic value of CDX2 has also been examined in malignancies; CDX2 was found to be expressed in gastric cancer, colon cancer, esophageal carcinoma, and other tumors such as intrahepatic intraductal papillary neoplasia (*25,30-35*). However, CDX2 expression and its prognostic significance in ICC has not been widely reported.

Accordingly, the current study immunohistochemically evaluated the expression of CDX2 and its prognostic value in ICC. In addition, the association between CDX2 expression and clinicopathological features of ICC was also examined.

2. Materials and Methods

2.1. *Patients and tumor samples*

The study was reviewed and approved by the Ethics Committee of the PLA General Hospital (Beijing, China). Subjects were enrolled over a 6-year period (from 2011 to 2016). Subjects were patients with pathologically confirmed ICC who underwent radical resection at the PLA General Hospital. To ensure the verifiability of this study, the following exclusion criteria were used when selecting patients: *i*) patients with additional malignancies in other organs or systems; *ii*) patients with a pathological diagnosis of combined hepatocellular carcinoma and cholangiocarcinoma; *iii*) evidence of cancer cells in the surgical margins; and *iv*) patients lost to follow-up. Patients meeting any of the four exclusion criteria were excluded. Demographic data were collected and clinicopathological features were examined in all patients enrolled in this study using a computerized medical database, perioperative records and pathology reports were thoroughly reviewed, and a supplementary follow-up was conducted by telephone.

2.2. *Immunohistochemistry*

Tissues were immunohistochemically stained using the Envision two-step method (*36*). Briefly, specimens were fixed in formalin and embedded in paraffin to prepare 4-μm-thick sections as previously described (*36*). Slides were serially incubated (40 min each, at room temperature) with CDX2 primary antibody (1:150, ZETA, USA). Each incubation with primary antibody was followed by 40-min of incubation at room temperature with horseradish peroxidase-labeled polymer. The stain was developed by incubation with diaminobenzidine substrate-chromogen for 5-10 min, and a hematoxylin counterstain was applied. Both negative and positive controls were used.

2.3. *Scoring of staining*

Staining was graded for intensity (0, negative; 1, weak; 2, moderate; and 3, strong), and the percentage of cells stained was determined (0, < 5%; 1, 6-25%; 2, 26-50%; 3, > 50%). The final score was calculated as the combined staining score (percentage of cells + intensity). A score less than 1 was defined as negative expression, and a score equal to or greater than 1 was defined as positive expression (*35*).

2.4. *Definition*

In the current study, hepatolithiasis was defined as concretions existing in the intrahepatic bile ducts; ICC due to hepatolithiasis refers to ICC as a result of hepatolithiasis, and ICC not due to hepatolithiasis refers to ICC not resulting from hepatolithiasis (*37-39*). Based on the macroscopic appearance described by the Liver Cancer Study Group of Japan, ICC consists of 3 gross subtypes: the mass-forming subtype, the periductal infiltrating subtype, and the intraductal growth subtype. Lymphatic invasion mostly refers to lymph node involvement at the site of the hepatoduodenal ligament (involvement of regional lymph nodes (N1 disease) according to the 8th edition of the American Joint Committee on Cancer Staging System).

2.5. *Statistical analysis*

Statistical analyses were performed using SPSS v14.0 (IBM, Armonk, NY, USA). Categorical variables are expressed as a percentage. Continuous variables are expressed as the mean and standard deviation. Categorical variables were compared using the χ^2 test or Fisher's exact test, as appropriate; continuous variables were compared using the Mann-Whitney *U* test. Long-term survival was estimated using the Kaplan-Meier method and compared using the log rank test. A two-tailed *p* value less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. *Demographic data and clinicopathological characteristics of patients*

Strict application of the exclusion criteria resulted in a

Table 1. Correlation between CDX2 expression according to immunohistochemistry and clinicopathological features of ICC

Items	Total	CDX2 immunohistochemistry expression		*p* value
		Positive	Negative	
Age (year, mean ± SD)		53 ± 7.8	51 ± 7.0	0.23[#]
Gender				0.78[&]
Male	60	18 (30.0%)	42 (70.0%)	
Female	33	9 (27.3%)	24 (72.7%)	
Tumor nodules				0.30[&]
Single	69	22 (31.9%)	47 (68.1%)	
Multiple	24	5 (20.8%)	19 (79.2%)	
Hepatolithiasis				0.02[&]
With hepatolithiasisPresent	63	23 (36.5%)	40 (63.5%)	
Without hepatolithiasisAbsent	30	4 (13.3%)	26 (86.7%)	
Gross subtype				0.63[*]
Mass-forming	55	18 (32.7%)	37 (67.3%)	
Peri-ductal infiltrating	13	2 (15.4%)	11 (84.6%)	
Intraductal papillary	25	7 (28.0%)	18 (72.0%)	
Lymphatic invasion				< 0.01[&]
PositivePresent	52	7 (13.5%)	45 (86.5%)	
NegativeAbsent	41	20 (48.8%)	21 (51.2%)	
Differentiation				0.01[*]
Well differentiated	12	5 (41.7%)	7 (58.3%)	
Moderately differentiated	21	10 (47.6%)	11 (52.4%)	
Poorly differentiated	60	12 (20.0%)	48 (80.0%)	
TNM Staging				0.72[*]
1	49	17 (34.7%)	32 (65.3%)	
2	27	6 (22.2%)	21 (77.8%)	
3	17	4 (23.5%)	13 (76.5%)	

CDX2: caudal homeobox 2; SD, standard deviation; [#], Student's *t* test; [*], Mann–Whitney *U* test; &, χ^2 test.

total of 93 patients with pathologically confirmed ICC in this study. Corresponding tissue samples (paraffin-embedded sections) were obtained from surgically resected specimens from the 93 patients. Demographic data and clinicopathological characteristics of patients are shown in Table 1. The 93 patients with ICC consisted of 60 males and 33 females with a mean age of 51.6 years (standard deviation: 7.3 years). Multiple tumor nodules were present in 24 of the 93 patients, and 63 patients had underlying hepatolithiasis. Fifty-five of the 93 patients had the mass-forming gross subtype, 13 had the peri-ductal infiltrating subtype, and 25 had the intraductal papillary subtype. Lymphatic invasion was present in 52 patients and absent in 41. Twelve patients had well-differentiated tumors, 21 had moderately differentiated tumors, and 60 had poorly differentiated tumors. Forty-nine tumors were T1, 27 were T2, and 17 were T3. The follow-up ranged from 6 months to 86 months in length.

3.2. *CDX2 expression according to immunohistochemistry*

CDX2 staining was immunohistochemically assessed in all 93 specimens of ICC. As shown in Figure 1, CDX2 protein was mainly concentrated in the nuclei of carcinoma cells. CDX2 staining was noted in 27 specimens (29.03%). Subjects were divided into patients with ICC due to hepatolithiasis and those with ICC not due to hepatolithiasis. Positive CDX2 expression was noted in 23 of 63 patients with ICC due to hepatolithiasis (36.51%) and in 4 of 30 patients with ICC not due to hepatolithiasis (13.33%). The rate of expression differed significantly in the two groups (χ^2 = 5.30, p = 0.02).

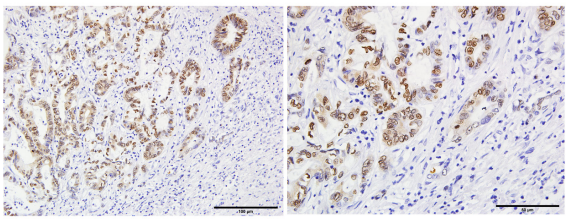

Figure 1. CDX2 expression in ICC. A Strong immunostaining of the nuclei of carcinoma cells in yellow or brown. (left, ×100; right, ×200)

3.3. *Survival analysis*

Patients were categorized into two groups, those with CDX2-positive tumors and those with CDX2-negative tumors. Patients with CDX2-positive tumors had a median survival of 40 months, whereas those with CDX2-negative tumors had a median survival of a mere 13 months; as shown in Figure 2, patients with CDX2-positive tumors had a significantly lower likelihood of dying compared to those with CDX2-negative tumors (hazard ratio (HR) 0.36, 95% confidence interval (95% CI) 0.22-0.59; p < 0.001).

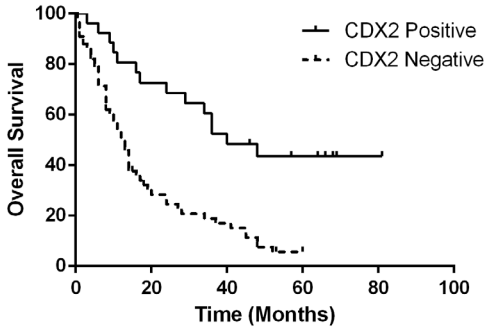

Figure 2. Kaplan-Meier survival analysis by CDX2 status. The y-axis represents the percentage of patients, and the x-axis represents their survival in months. The solid line represents patients with CDX2-positive tumors, who tended to survive longer than patients with CDX2-negative tumors represented by the dotted line ($p < 0.001$).

3.4. *Correlation between CDX2 expression and clinicopathological features*

The current study noted a significant correlation between CDX2 expression and lymphatic invasion; the rate of CDX2 expression was 13.46% in patients with lymphatic invasion and 48.78% in patients without lymphatic invasion ($\chi^2 = 13.88$, $p < 0.01$). In addition, positivity for CDX2 expression was significantly higher in patients with well-differentiated or moderately differentiated tumors than that in patients with poorly differentiated tumors (41.7% in patients with well-differentiated tumors, 47.6% in patients with moderately differentiated tumors, and 20.0% in patients with poorly differentiated tumors; Mann-Whitney U test, $p = 0.01$). There were no statistical differences between positive CDX2 staining and gender, the number of tumor nodules (single or multiple), tumor gross subtype, and TNM stage.

The prognostic value of CDX2 has been noted in relation to certain malignancies. However, its predictive significance in ICC has yet to be determined. The current study is as a relatively large-scale study that immunohistochemically evaluated the expression of CDX2 and its prognostic value in predicting ICC. Results indicated that patients with CDX2-positive tumors had significant survival advantages over those with CDX2-negative tumors (HR 0.36, 95% CI 0.22-0.59). Moreover, CDX2 staining was positive in 29.03% of the patients with ICC, and this finding agrees with figures reported in the literature (*40-42*). A correlation between CDX2 expression and the presence of hepatolithiasis, the presence of lymphatic invasion, and the extent of tumor differentiation was also noted. Interestingly, expression differed significantly in patients with ICC due to hepatolithiasis and patients with ICC not due to hepatolithiasis (36.51% *vs*. 13.33%, $\chi^2 = 5.30$, $p = 0.02$). Taken together, these findings suggest that CDX2 might serve as a marker of prognostic significance for patients with ICC in the future.

The detailed mechanisms that account for the correlation between CDX2 expression and survival disadvantages in patients with ICC have not been specifically studied. The following could be confounding variables.

Studies have indicated that positive expression of CDX2 is related to inhibition of invasion and metastasis by ICC, and CDX2 is considered to be an independent indicator of improved long-term survival (*41,43*). As reported previously, 16.46% of patients with biliary tract carcinoma had positive expression of CDX2 (*41*). In addition, a study has indicated that CDX2 was expressed in 37.3% of extrahepatic biliary tract carcinoma and more frequently in tumors with papillary growth and no vascular invasion (*42*). Similar results were reported by Jinawath *et al*. (*33*). A study by Li *et al*, reported that expression of CDX2 was negatively correlated with tumor size and lymph node metastasis in biliary tract tumors; increased CDX2 expression was correlated with a better prognostic outcome (*40*). Similarly, the rate of positive CDX2 expression was 29.03% in the current study; CDX2 expression was also negatively associated with lymphatic invasion, but there was no significant relationship between CDX2 expression and morphological tumor type or TNM staging. Such discrepancies could be due to multiple factors, such as patient selection bias. CDX2 expression is correlated with a greater degree of differentiation in gastric and gallbladder adenocarcinoma (*29,40,44*), and the current study yielded a similar finding. Taken together, the aforementioned mechanisms or hypotheses might explain the current finding that patients with CDX2-positive tumors had superior survival.

That said, the rate of CDX2 expression in patients with ICC due to hepatolithiasis was significantly higher than that in patients with ICC not due to hepatolithiasis. This finding implies the possible involvement of CDX2 in the pathogenesis of hepatolithiasis leading to ICC. Hepatolithiasis was deemed to be a confirmed risk factor for ICC. Mucin hypersecretion or aberrant expression of mucin 2 and mucin 5AC in the intrahepatic biliary system is closely associated with the lithogenesis of hepatolithiasis (*45*). Two studies in Japan confirmed that aberrant expression of CDX2 is closely correlated with the overexpression of mucin 2 or mucin 5AC in mucinous ICC due to hepatolithiasis, suggesting its role in intestinal differentiation and its association with carcinogenesis in these tumors (*31,33*). One might conclude that patients with ICC due to hepatolithiasis had a higher rate of CDX2 expression. However, the detailed mechanism for this expression needs to be studied further.

The current study has several limitations. The detailed or definitive molecular mechanisms accounting for the correlation with CDX2 expression were not explored, thus reducing the reliability and consistency of the current results. Moreover, CDX2 expression was

not examined in patients with hepatolithiasis alone, and those patients could serve as a control or parallel group. Nevertheless, the current study has helped to lay out a persuasive argument for the prognostic significance of CDX2 in ICC.

In conclusion, positive CDX2 expression resulted in significant survival advantages in ICC. CDX2 might be used as a prognostic marker in patients with ICC.

Acknowledgements

This work was supported by a grant from the Beijing Natural Science Foundation (no.7172201), a grant from the National Natural Science Foundation of China (NSFC) (no.81502376), and grants from the Beijing Nova program in Science and Technology (no. xx2018063) and the Foundation for Outstanding Youth in PLA General Hospitals (to Meng X).

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(Received April 19, 2018; Revised May 6, 2018; Accepted May 20, 2018)

Brief Report

DOI: 10.5582/irdr.2018.01052

Current status of malignant mesothelioma with liver involvement in China: A brief report and review of the literature

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Summary **Primary and secondary intrahepatic malignant mesothelioma (PIHMM & SIHMM) caused by Peritoneal mesothelioma (PM) are extremely rare tumors and their clinicopathological characteristics remain unclear. The current study presented a case of a 63-year-old female with PIHMM and a literature review of Chinese case reports of SIHMM and PIHMM was performed. The patient received curative left hemihepatectomy because of a 5.5 × 5.0 × 4.0 cm mass occupying the II, III and the lateral portion of the IV segments and meanwhile tightly infiltrating the diaphragm (yellow arrow) was also observed. The pathological diagnosis was epithelial type PIHMM. Immunohistochemistry revealed that the tumor was positive for Calretinin, CK5/6, WT-1 and D2-40(N). The literature review included 11 studies and 6 case reports with a total of 293 PM patients accompanied with 31 SIHMM cases and then 3 case reports of PIHMM. SIHMM and PIHMM are extremely rare, easy to misdiagnose malignant tumors. Immunohistochemistry should be performed strictly in accordance with guidelines, which is crucial for pathological diagnosis. Comprehensive treatment of surgery combined with chemotherapy are mainstream methods for SIHMM and PIHMM. Also, exact survival data should be carefully explored so that objective evaluation of the efficacy of the treatment could be achieved.**

Keywords: Malignant mesothelioma, liver involvement, China

1. Introduction

Malignant mesothelioma (MM) are tumors of the mesothelial cells which usually arise from the pleura, peritoneum, pericardium and occasionally, the tunica vaginalis. Approximately 20-35% of all the MM are peritoneal, just next to pleural mesothelioma (60-65%) (*1-4*). Peritoneal mesothelioma (PM) has characteristics of constant invasion of the adjacent visceral organs but infrequent metastasis to the liver (*5-7*). Besides secondary intrahepatic malignant mesothelioma (SIHMM), primary intrahepatic mesothelioma (PIHMM) was also reported with only less than 20 case reports from around the world so far, and is not yet included in the World Health Organization (WHO) classification of hepatic tumors (*8*). Therefore, PIHMM and SIHMM are too easy to misdiagnosis as HCC or metastatic liver tumors in routine clinical work.

Notably, a considerable proportion of case reports of PIHMM and SIHMM come from China. However, until now, there is still lack of a systematic summary of this rare disease in China regarding etiology, epidemiology, diagnosis, pathology and treatment. The present study provides a case report accompanied with a detailed literature review of Chinese reports, which aims to raise the awareness and improve the quality of therapeutic effects for the extremely rare PIHMM/SIHMM.

2. Materials and Methods

The current study presented a case of a 63-year-old female with PIHMM and a literature review of Chinese case reports of SIHMM and PIHMM was performed. The study was approved by the ethics committee of XinHua Hospital affiliated to Shanghai JiaoTong

Released online in J-STAGE as advance publication May 25, 2018.

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Two investigators (DZ and WJD) performed the literature search independently by using Pubmed, Embase, ISI and "CNKI (Chinese)", "WANFANG (Chinese)", "WEIPU (Chinese)" databases between January 1970 and April 2018. The search was limited to humans. The search strategy was based on the following English Medical Subject Heading terms (MeSH) and its correspondent Chinese text words: "mesothelioma", "malignant mesothelioma", "peritoneal mesothelioma", "intrahepatic mesothelioma", "primary intrahepatic mesothelioma", "secondary intrahepatic malignant mesothelioma", "SIHMM", "PIHMM". The related article's function and reference lists were used to broaden the search. The investigators and experts in this field ensured that all potentially relevant reports were identified. No restriction was set for languages or date of publication. When further information was required, the corresponding authors of relevant papers were contacted by the reviewers.

3. Results and Discussion

3.1. *Characteristics of the patient*

A 63-year-old female presented with upper abdominal pain that occurred when she rolled over during her sleep for half a year and was admitted to Department of General Surgery, XinHua Hospital affiliated to Shanghai JiaoTong University, School of Medicine in March, 2017.

She has no history of asbestos exposure or special pathogen infection. Laboratory examinations revealed no abnormal results concerning the blood routine index, liver and renal function, or tumor markers. Viral markers related to hepatitis B virus (HBV), hepatitis C virus (HCV), syphilis, or human immunodeficiency virus (HIV) infection were all negative. The gastroscopy examination showed a flat bulge of about 2.5 × 2.3 cm with a smooth surface mucous membrane in the upper part of body of the stomach (Figure 1A). The endoscopic ultrasonography (EUS) confirmed the above lesion as an external compression caused by the left lobe of the liver (Figure 1B). Several hypoechoic areas with clear boundary and inhomogenous internal echoes in the left-lateral lobe were detected by abdominal ultrasound. Further color Doppler flow image (CDFI) detected no blood flow signal in this lesion. The MRI revealed a 6.4 × 4.0 cm mass occupying the left-lateral hepatic lobe presenting an unclear boundary with the front edge of the stomach. The mass was shown as hypo-intensity on T1WI, while slightly hyper-intensity on T2WI signals (Figure 1C and 1D). Abnormal conditions were not found in pancreas, gallbladder, spleen, adrenals, kidneys, bowel loops and the pelvic cavity. There was no evidence of ascites, pleural effusion, thickening or a peritoneal malignant

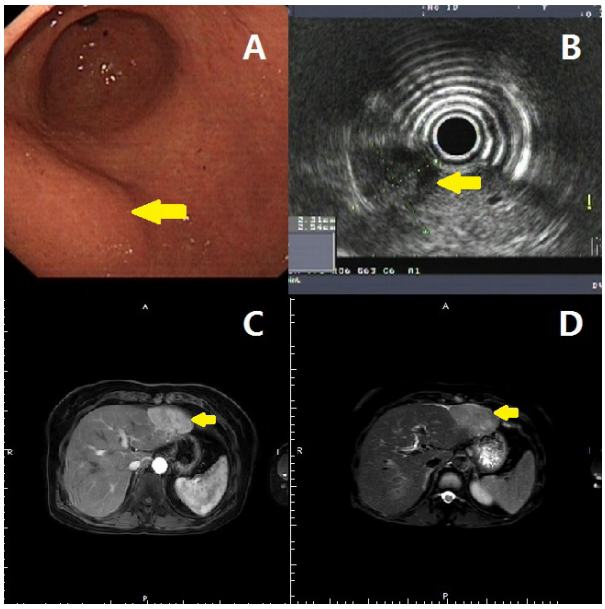

Figure 1. Diagnosis of the present case of PIHMM. Gastroscopy showed a flat bulge of about 2.5 × 2.3 cm with a smooth surface mucous membrane in the upper part of body of the stomach (yellow arrow) **(A)**. The endoscopic ultrasonography (EUS) confirmed the above lesion as a external compression caused by the left lobe of the liver (yellow arrow) **(B)**. The MRI revealed a 6.4 × 4.0 cm mass occupying the left-lateral hepatic lobe presenting an unclear boundary with the front edge of the stomach. The mass was showed as hypo-intensity on T1WI and peripheral serpiginous vessels were shown (yellow arrow) **(C)**, while slightly hyper-intensity on T2WI signals (yellow arrow) **(D)**.

tumor. The suspected diagnosis of Focal Nodular Hyperplasia (FNH) or exogenic gastric stromal tumor (GIST) was made by the MRI radiological doctor.

The patient then received an exploratory laparotomy. There was no significant ascites or metastasis sign in the abdominal cavity. A soft, 5.5 × 5.0 × 4.0 cm tumor occupying the II, III and the lateral portion of the IV segments and meanwhile tightly infiltrating the diaphragm was also observed (Figure 2A, 2B and 2C). No enlarged lymph node was detected in the hepatoduodenal ligament (HDL), around the stomach, retroperitoneum or pelvics cavity except a 5.5 × 5.0 × 4.0 cm nodule was found beneath the diaphragma. Therefore, complete resection of the left hepatic lobe was performed. Hepatic portal occlusion utilizing the Pringle's maneuver was conducted twice for 5 and 13 minutes, respectively. The beneath diaphragma nodule was also resected and a negative margin was obtained. The intraoperative blood loss was approximately 300 mL and a drainage tube was placed into the foramen of Winslow before abdominal closure.

No complications including postoperative bleeding, liver dysfunction or bile leakage occurred and the drainage tube was removed at the 7th postoperative day (7 POD). The patient was discharged uneventfully at 10 POD. The pathological diagnosis was epithelial type PIHMM and surgical margins were free of tumor (Figure 2D and 2E). The beneath diaphragma nodule

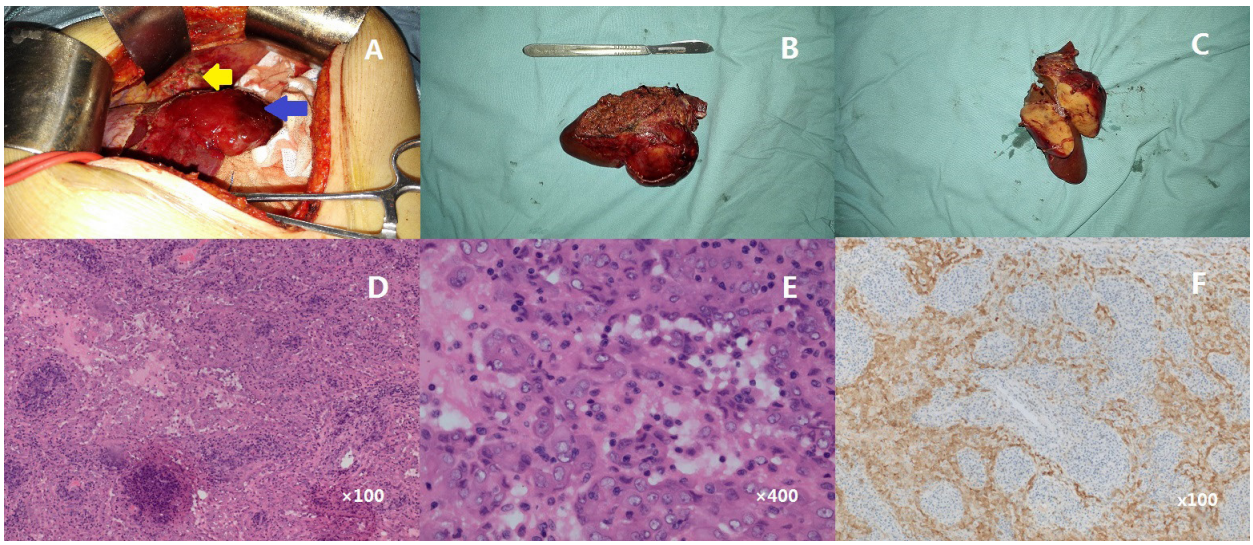

Figure 2. During the operation, a 5.5 × 5.0 × 4.0 cm tumor (blue arrow) occupying the II, III and the lateral portion of the IV segments while tightly infiltrating the diaphragm (yellow arrow) was observed (A). The sample of the removed PIHMM (B). Section plane of the tumor sample (C). HE staining (×100) (D) and (×400) (E) of the tumor sample. Calretinin staining of the tumor (×100) (F).

was eventually diagnosed as cyst with lymphoid tissue hyperplasia. Immunohistochemistry revealed that the tumor cells were positive for CK7, CK19, Calretinin (Figure 2F), AE1/3, CK8, CD34, a1-AT(N), INI-1(N), B-cat(N), CyclineD1, CA125, DES, MC, WT-1(N), D2-40(N), F8(N), CD138, CD163, CD38, LCA, kp1 and partial positive for VIM, CD31, Ki-67 (10%+), CK5/6, Kappa, Lamda, S100, and totally negative for CEA, CK20, AFP, Hepa(N), HBsAg(N), HBcAg(N), HMB45(N), Glypican-3(N), TTF1, P53, TTF1, E-CAD, NapsinA, CK5, MUM1(N), SMA, CGA and SYN.

No adjuvant chemotherapy was given and the patient was disease-free survival at 13 months follow-up.

The literature review included 11 studies and 6 case reports with a total of 293 Chinese PM patients who received treatment from 1970~2016 among which 31 were patients of SIHMM (*9-25*). Then, three case reports with 3 cases of PIHMM were also included (*26-28*).

Characteristics of the included patients of SIHMN caused by PM and PIHMN plus our present case are listed in Table 1 and Table 2, respectively. The imaging and pathological diagnostic information of the included patients of localized SIHMN and PIHMN are listed in Table 3.

3.2. *Epidemiology*

Peritoneal mesothelioma (PM) is a rare malignancy, with an incidence of 0.1~0.2/100,000 in China. He Bei Province and Da Yao in Yun Nan Province are the two high prevalence areas of PM due to large numbers of asbestos industrial factories in the past 40 years (*29,30*). The male/female ratio and median age at the initial diagnosis were reported to be 2:1~3:2 and 45~70 years, respectively, but varies a lot between different regions. The prognosis of PM is poor with a meso life span of about 1.5 years even after combined therapy (*31*).

The incidence of SIHMM caused by PM, including the diffuse type and the localized type of gross pathological classification, is approximately 3.8% in China and most of the diagnosed SIHMM belong to the former (*32*). Table 1 lists a total of 293 diffuse type PMs accompanied with 31 SIHMM cases among which 22 and 9 were metastatic and invasion patients. Then, only 10 Chinese cases of localized SIHMM have been reported and there is still no coresponding published data in other countries. Until now, no clear risk factors for liver involvement caused by SIHMM have been identified and there are also no differences between age, gender, the onset and the prognosis when comparing the two types of SIHMM.

PIHMM is even a rarer type of mesothelioma than SIHMM. To the best of our knowledge, only 15 cases of PIHMM have been previously reported worldwide in the published literature and 3 of them come from China (*26-28*). The characteristics of these 3 cases plus our present case are listed in Table 2. The age range of these patients were 24~63 (average of 45.75). However, contrary to the published reports in other countries, the male/female ratio of the 4 cases was 1:3.The OS of PIHMM was reported to range from 2 to 24 months whereas among these 4 Chinese patients only the present case provided follow-up data and the patient is still alive after she received surgery 13 months ago.

3.3. *Etiology*

Asbestos, erionite and vacuolating virus 40 (SV40) are known as the three already known risk factors for mesothelioma (*33*). Previous study showed that

Table 1. Characteristics of the included patients of SIHMN caused by PM

Author	Year	Type of PM	No. Of SIHMN/PM	Gender	Age (yr)	Etiology			Invasion to Liver	Metastasis to Liver	Treatment	OS
						Asbestos	Erionite	SV40				
Zhang *et al.* (*9*)	1973~2002	D	3/41	32M/9F	25~76	0/41	N.M	N.M	-	√	Op+Chemo	16.5 mo
Kang *et al.* (*10*)	1980~2012	D	5/20	13M/7F	14~79	0/20	N.M	N.M	-	√	Op+Chemo	9 mo
Guo *et al.* (*11*)	1985~2004	D	2/32	23M/9F	28~76	9/32	N.M	N.M	-	√	Op+Chemo	3 mo ~ 5 yr
Tong *et al.* (*12*)	1995~2015	D	8/21	17M/4F	23~87	1/21	N.M	N.M	-	√	Op+Chemo	3 mo ~ >2 yr
Pan *et al.* (*13*)	2000~2009	D	3/15	11M/4F	35~76	0/15	N.M	N.M	-	√	Chemo	3 ~ 18 mo
Shan *et al.* (*14*)	2000~2010	D	1/1	F	45	1/1	N.M	N.M	√	-	Op+Chemo	8 mo
Zhou *et al.* (*15*)	2001~2011	D	1/17	6M/11F	13~77	0/17	N.M	N.M	-	√	Op+Chemo	1 mo ~ 12 yr
Li *et al.* (*16*)	2003~2013	D	1/25	12M/13F	38~78	0/25	N.M	N.M	√	-	Op+Chemo	4 ~ 21 mo
Wang *et al.* (*17*)	2004~2010	D	4/5	4M/1F	54~73	0/5	N.M	N.M	√	-	Op+Chemo	7mo
Zhou *et al.* (*18*)	2005~2011	D	2/16	10M/6F	28~65	0/16	N.M	N.M	√	-	Op+Chemo	1 mo ~ >12 yr
An *et al.* (*19*)	2009~2013	D	1/100	70M/30F	3~83	0/100	N.M	N.M	√	-	N.M	N.M
Dong *et al.* (*20*)	2005	L	1/1	M	55	0/1	N.M	N.M	-	√	N.M	N.M
Zhang *et al.* (*21*)	2008	L	1/1	M	49	0/1	N.M	N.M	√	-	Chemo	N.M
Shan *et al.* (*14*)	2000~2010	L	4/4	1M/3F	51~69	4/4	N.M	N.M	√	-	Op+Chemo	5 ~ 8 mo
Zhao *et al.* (*22*)	2012	L	1/1	F	64	0/1	N.M	N.M	-	√	Op+Chemo	6 mo
Gao *et al.* (*23*)	2012	L	1/1	M	46	0/1	N.M	N.M	-	√	RFA	N.M
Li *et al.* (*24*)	2015	L	1/1	M	69	0/1	N.M	N.M	-	√	N.M	N.M
Zhong *et al.* (*25*)	2016	L	1/1	M	69	0/1	N.M	N.M	√	-	Op	N.M

SIHMN: secondary intrahepatic malignant mesothelioma; PM: D: diffuse type; L: localized type; M: Male; F: Female; Op: operation; Chemo: Chemotherapy; RFA: radiofrequency ablation; mo: month(s); yr: year(s); OS: overall survival; N.M: not mentioned.

Table 2. Characteristics of the included patients of PIHMN

Author	Year	No. of PIHMN Patient	Gender	Age	Etiology			Location of PIHMN	Size of PIHMN (cm)	Treatment	OS
					Asbestos	Erionite	SV40				
Yan *et al.* (*26*)	2001	1	F	46	0/1	N.M	N.M	Left lateral lobe/Left medial lobe	10 × 5.5/11 × 10	Op	N.M
Wang *et al.* (*27*)	2003	1	M	24	0/1	N.M	N.M	Right lobe	7 × 6 × 6	Op	N.M
Dong *et al.* (*28*)	2014	1	F	50	0/1	N.M	N.M	Right and left lobe	N.M	Op	N.M
Present case	2017	1	F	63	0/1	N.M	N.M	Left lobe	5.5 × 5.0 × 4.0	Op	13 mo, Still alive

PIHMN: primary intrahepatic malignant mesothelioma; M: Male; F: Female; Op: operation; mo: month(s); yr: year(s); OS: overall survival; N.M: not mentioned.

Table 3. Imaging and pathological diagnostic information of the included patients of localized SIHMN and PIHMN

Author	Type	Imaging Methods for Diagnosis				Misdiagnosis	Pathological Type			Immunohistochemistry					
		US	CT	MRI	PET-CT		Epithelioid	Sarcomatoid	Biphasic	D2-40	WT-1	Calretinin	CK5/6	AFP	CK7	Vimentin
Dong *et al.* (*20*)	S	√	√	√	N.E	HCC	N.E	N.E	N.E	N.E	N.E	N.E	N.E	N.E	N.E	N.E
Zhang *et al.* (*21*)	S	N.E	N.E	√	N.E	N.M	N.E	N.E	N.E	N.E	N.E	N.E	N.E	N.E	N.E	N.E
Zhao *et al.* (*22*)	S	N.E	√	N.E	√	HCC	-	√	-	+	N.E	+	+	-	+	N.E
Gao *et al.* (*23*)	S	√	N.E	N.E	N.E	CRLM	N.E	N.E	N.E	N.E	+	N.E	-	N.E	-	+
Li *et al.* (*24*)	S	N.E	√	N.E	N.E	Sarcoma	N.E	N.E	N.E	N.E	N.E	N.E	-	-	N.E	+
Zhong *et al.* (*25*)	S	N.E	N.E	√	N.E	HCC	N.E	N.E	N.E	N.E	N.E	Partial +	+	N.E	-	N.E
Shan *et al.* (*14*)	S	N.E	√	N.E	N.E	No	√	-	-	N.E	N.E	+	N.E	N.E	N.E	N.E
Shan *et al.* (*14*)	S	N.E	√	N.E	N.E	No	√	-	-	N.E	N.E	+	N.E	N.E	N.E	N.E
Shan *et al.* (*14*)	S	N.E	√	N.E	N.E	No	√	-	-	N.E	N.E	+	N.E	N.E	N.E	N.E
Shan *et al.* (*14*)	S	N.E	√	N.E	N.E	No	√	-	-	N.E	N.E	+	N.E	N.E	N.E	N.E
Yan *et al.* (*26*)	P	√	√	N.E	N.E	Echinococcosis	-	-	√	N.E	N.E	N.E	N.E	N.E	N.E	+
Wang *et al.* (*27*)	P	√	√	√	N.E	AML	√	-	-	N.E	N.E	N.E	N.E	N.E	N.E	N.E
Dong *et al.* (*28*)	P	N.E	√	N.E	√	HCC	√	-	-	N.E	N.E	+	N.E	N.E	N.E	+
Present case	P	N.E	N.E	√	N.E	FNH/GIST	√	-	-	+	+	+	Partial +	-	+	Partial +

SIHMN: secondary intrahepatic malignant mesothelioma; PIHMN: primary intrahepatic malignant mesothelioma; S: secondary intrahepatic malignant mesothelioma; P: primary intrahepatic malignant mesothelioma; WT-1: Wilm's tumor-1; CK: cytokeratin; AML: angiomyolipoma of liver; ,CRLM: colorectal liver metastasis; HCC: hepatocarcinoma; FNH: focal nodular hyperplasia; GIST: gastrointestinal stromal tumor; N.E: not evaluated; N.M: not mentioned.

41.7~86.8% of the PM patients had a history of asbestos exposure (*34-36*). However, in the included studies, the rate of clear contact history of asbestos were only 3.75% (11/293), 40.00% (4/10) and 0.00% (0/4) among the diffuse SIHMM, localized SIHMM and PIHMM patients. In fact, the role of asbestos in the occurrence of PM remains to be debated. Some pathologists thought that asbestos exposure has no value in pathological differential diagnosis of mesothelioma although the role of asbestos in malignant mesothelioma pathogenesis is already confirmed by rat models. Amphibole asbestos, particularly crocidolite, has been reported to be a much more potent agent that causes PM than serpentine asbestos. However, the latter remains as a general agreement of agent and accounts for over 95% of the asbestos used around the world (*14*). Alarmingly, as the incubation period from the first exposure to asbestos to the occurrence of mesothelioma generally needs 20~40 years, this important risk factor might be overlooked in routine practice of this easily misdiagnosed disease.

In patients with no history of asbestos exposure, post-mortem examination and animal experiments showed that 30~50% might be associated with SV40 infection (*37*). Some other authors believe SV40 might act as a cooperative factor with asbestos and enhance its pathogenic effect. The mesothelioma-inducing role of erionite was also confirmed by rat models but unfortunately, SV40 and erionite might not be familiar to the majority of doctors and no related contact history of the above two agents could be provided in the listed Chinese case reports as well as literature from other countries.

3.4. *Imaging diagnosis*

Due to the rarity of mesothelioma and less radiology experience, a correct preoperative diagnosis rate of SIHMM and PIHMM is extremely low around the world. Among the 14 cases listed in Table 3, only 4 cases (28.57%) from one institute clearly declared they avoided misdiagnosis before surgery.

CT and MRI are the most frequent utilized techniques for diagnosing this tumor but the radiologic features of mesothelioma with liver involvement have not yet been clearly defined. Since hemorrhage and necrosis are very common pathological changes in SIHMM and PIHMM, In CT scan, hyper-dense components caused by hemorrhage and hypo-densities, especially in a central area, might be the necrosis lesion of PIHMM. For SIHMM patients, the CT images might indicate thickened peritoneum and omentum surrounding the liver, accompanied with an irregular nodular tumor infiltrating into the liver surface (*38*).

On MRI, heterogeneous hyper-dense areas of hemorrhage can be detected in T1-weighted images. The necrotic components among the solid tumor might show up as multiple cystic structures with irregular

internal septations. Contrast-enhancement is useful for detecting PIHMM. On post-contrast images, typical signs of PIHMM include peripheral serpiginous vessels, as well as a septal and increasing enhancement pattern from the periphery to the middle part of the tumor on delayed phase (*39*). However, a considerable number of cases do not have typical imaging features mentioned above (Figure 1C and 1D).

There were 2 listed reports, which utilized PET-CT for diagnosing SIHMM and PIHMM, respectively. However, both of them still made a misdiagnosis of hepatocarcinoma (HCC). The reason might be that although PET-CT could clearly reveal high FDG uptake in the intrahepatic tumor, no significant FDG accumulation was noted in omentum or peritoneum, thus resulting in insufficient differentiation effectiveness with other liver tumors (*28*). Coincidentally, hypermetabolic peripheral regions and internal septations of PIHMM might also be easily misdiagnosed as hepatic cystadenocarcinoma.

3.5. *Pathology and immunohistochemistry*

In gross pathology, SIHMM caused by PM could be divided into the diffuse type and the localized type. The diffuse type is generally presented as dark red or gray-white nodules of varying sizes on the peritoneum often accompanied with extensive adhesions with liver and other organs, which finally induce the "frozen" abdominal cavity. The localized type, however, is featured as multiple independent nodular lesions or accumulated masses located on the surface of liver and peritoneum (*40*). Ascites is the most common manifestation of PM, but it is also the major reason for misdiagnosis due to its lack of specificity and is easily confused with tuberculous peritonitis and primary, secondary or peritoneal metastatic liver tumors.

Histologically, PM includes three types: epithelial, sarcomatoid, and biphasic. Epithelial is the most common type and there were 7 cases (4 SIHMMs and 3 PIHMMs, respectively) among our listed case reports presented as epithelial type. The biphasic type has a mixture of epithelioid and sarcomatous components and each component accounts for at least 10% of the tumor.

Immunohistochemistry is the most crucial technique for pathological diagnosis of SIHMM and PIHMM. The International Mesothelioma Interest Group (IMIG) recommended that any combination of markers should contain at least 2 mesothelioma markers and 2 other cancer-related markers. Calretinin (Calcinein), CK5/6 (cytokeratin5/6, cytokeratin), WT-1, and D2-40 (Dodoplanin, peduncle) are considered to be the best markers for differential diagnosing mesothelioma (*40,41*). Calretinin is a calcium-binding protein commonly expressed in nerve, adipose, mesothelial, and very few adenocarcinoma tissues. Calretinin has been regarded as the most specific and sensitive antibody for detecting epithelioid malignant mesothelioma. CK5/6, as a member of cytokeratins, is generally expressed in mesothelial squamous as well as transitional tissues, and occasionally, in certain adenocarcinoma cells. In a previous report from China containing 100 cases of diffuse type of malignant mesothelioma, the positive rate of Calretinin and CK5/6 were 93% and 79.7%, respectively. D2-40 is a monoclonal antibody, which is selectively expressed in lymphatic endothelium, lymphoid tissue-derived tumors and cancer-infiltrated lymphatic vessels. WT-1 is a DNA binding transcription factor which is localized in the nucleus. Positive staining of WT-1 is useful for detecting nephroblastoma, connective tissue-proliferating small round cell tumors, Mullerian serous carcinoma and mesothelioma. The sensitivity and specificity of D2-40 and WT-1 are significantly lower than Calretinin and CK5/6. Notably, the vast majority of the data concerning the diagnostic effectiveness of the former 4 markers came from the diffuse type of malignant mesothelioma. In our listed case reports, there were only 57.14% (8/14), 28.57% (4/14), 14.29% (2/14) and 14.29% (2/14) of the patients performed Calretinin, CK5/6, WT-1 and D2-40 immunohistochemistry examination, although their positive rate was 100% (8/8), 75% (3/4), 100% (2/2) and 100% (2/2), respectively. This might reflect that SIHMM and PIHMM were really rare diseases even for pathologists in China.

3.6. *Treatment and prognosis*

The treatment for PM includes intravenous chemotherapy, intraperitoneal hyperthermic perfusion chemotherapy, cytoreductive surgery and surgical resection. Surgery is the best treatment for localized SIHMM and PIHMM, even for limited recurrence tumors. Unfortunately, there has been few prognosis data of SIHMM and PIHMM due to most of the papers were cases reports without follow-up information. The mean survival time was reported to be 5~8 months for SIHMM and the present case of PIHMM from our center had 13 months of disease-free survival after curative left hemihepatectomy. For the diffuse type of SIHMM, radical resection is impossible to achieve and is not advocated due to the fact that mesothelioma can infltrate the entire peritoneal cavity and the scars of laparotomy will cause tumor spread (*42,43*). Therefore, effective cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy (HIPEC) is beneficial to prolong the survival of these patients.

HIPEC can increase the local drug concentration because of its extensive contact with the tumor located in the plasma membrane and peritoneum. Pemetrexed combined with cisplatin is so far considered the first choice and standard protocol for inoperable peritoneal mesothelioma (*44*). For PM patients, a median survival of 31~34 months after cytoreductive surgery combined

with HIPEC as well as a 2-year survival rate of 79% after complete resection combined with HIPEC have been reported (*45*). In our listed case reports, the overall survival (OS) of PM patients was 1 month to over 12 years. However, clear statistics of the corresponding data specially for patients of the diffuse type of SIHMM are not available yet.

In conclusion, although the sheer number of SIHMM and PIHMM are greater in China than that in other countries in the world, they are still extremely rare, and it is easy to misdiagnose malignant tumors. The atypical imaging features and insufficient experience hinder radiologists from obtaining the correct diagnosis. Immunohistochemistry should be performed strictly in accordance with IMIG guidelines, which is crucial for pathological diagnosis. Comprehensive treatment of surgery combined with chemotherapy are mainstream methods for SIHMM and PIHMM but the prognosis is still not satisfactory. Also, exact survival data should be carefully explored so that objective evaluation of the efficacy of the treatment can be achieved.

Acknowledgements

The authors thank Dr.Wenbin Guan for his Immunohistochemistry examination for the patient. This study was supported by Grants from the National natural science foundation of China. No.:81401932.

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(Received May 3, 2018; Revised May 21, 2018; Accepted May 22, 2018)

Case Report

DOI: 10.5582/irdr.2018.01003

Muscular and cardiac manifestations in a Duchenne-carrier harboring a *dystrophin* deletion of exons 12-29

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Summary **Female carriers of mutations in the dystrophin gene (DMD-carriers) may manifest clinically in the skeletal muscle, the heart, or both. Cardiac involvement may manifest before, after, or together with the muscle manifestations. A 46y female developed slowly progressive weakness of the lower and upper limbs with left-sided predominance since age 26y. Muscle enzymes were repeatedly elevated and muscle biopsy showed absence of dystrophin. MLPA analysis revealed a deletion of exons 12-29. After starting steroids at age 39y, she developed palpitations and exertional dyspnoea. Cardiac MRI at age 41y revealed mildly reduced systolic function, a slightly enlarged left ventricle, mild hypokinesia of the entire myocardium, and focal, transmural late gadolinium enhancement (LGE) of the midventricular lateral wall. She did not tolerate beta-blockers but profited from ivabradine and lisinopril. In conclusion, muscle manifestations in DMD-carriers with deletions of exons 12-29 may start years before cardiac involvement becomes clinically apparent. Progressive worsening of systolic function in DMD-carriers is attributable to progressive myocardial fibrosis, as demonstrated by LGE. Steroids may trigger the development of cardiac disease in DMD-carriers.**

Keywords: Duchenne muscular dystrophy, cardiac involvement, heart failure, dystrophin, X-chromosomal, carrier, myopathy

1. Introduction

Due to skewed X-chromosome inactivation of one of the two X-chromosomes, female carriers of X-chromosomal gene mutations may present with variable clinical manifestations, ranging from normal to a phenotype like in males (*1*). This is also the case for carriers of deletions or point mutations in the *dystrophin* gene causing Duchenne muscular dystrophy (DMD) (DMD-carriers) (*1*). DMD-carriers are usually asymptomatic. However, some of the DMD-carriers become symptomatic and develop a progressive DMD-like phenotype. This is the case if the majority of the X-chromosomes carrying the wild-type allele is inactivated, The higher the percentage of inactivated wild-type *dystrophin* genes, the more severe will the phenotype of manifesting DMD-carriers be. Some of the DMD-carriers may even resemble male patients with DMD (*1*). Since *dystrophin* mutations not only manifest in the skeletal muscles but also in the myocardium, DMD males and DMD-carriers may develop variable degrees of cardiac disease (*2*). Cardiac involvement in DMD usually starts with ECG abnormalities until patients develop dilated cardiomyopathy. In DMD-carriers dilated cardiomyopathy may be the initial manifestation of the skewed X-inactivation. Here we present a DMD-carrier with muscle and myocardial manifestations since adulthood and compare them with clinical and genetic findings of previous reports.

2. Case Report

The patient is a 46y female, height 172 cm, weight

Released online in J-STAGE as advance publication April 15, 2018.

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64 kg, with an uneventful history until age 25y, when she developed difficulties climbing stairs. Muscle enzymes were repeatedly elevated (Table 1). During the further course she developed slowly progressive proximal muscle weakness with left-sided predominance and experienced recurrent falls without losing consciousness. The patient was first admitted to the authors'institution at age 29y. Work-up for the above mentioned abnormalities at age 29y with informed consent of the patient revealed scapula alata, weakness (M4-) and wasting of the shoulder girdle and proximal upper limb muscles, and proximal weakness and reduced tendon reflexes of the lower limbs. Muscle biopsy at age 29y from the right deltoid muscle revealed myopathic features with atrophic and hypertrophic muscle fibres, necrotic fibres, proliferation of endomysial connective tissue (Figure 1A), absence of dystrophin on the sarcolemma of some fibres (mosaic pattern) (Figure 1B), and upregulation of utrophin (Figure 1C). At age 37y genetic testing of blood lymphocyte DNA by means of multiplex ligation-dependent probe amplification (MLPA) revealed a heterozygote deletion of exons 12-29 in the dystrophin gene (NM_004006.2:c.(1331+1_1332-1)_(4071+1_4072-1)del). According to the Leiden DMD reading frame checker this mutation leads to an out-of-frame deletion (*http://www.dmd.nl/*). At age 39y she had experienced a right-sided tibial shatter fracture after a fall when going down a stair. At age 43y she underwent surgery for left-sided clubfoot. She was regularly smoking 20 cigarettes/d since age 18y. The family history was positive for death from DMD at age 15y (nephew), myocardial infarction (mother), lung carcinoma (father), and uterus carcinoma (sister) (Figure 2). She had been taking aprednisolon (initially 25mg/d after some weeks 5mg/d) between age 39y and age 43y.

Clinical neurologic examination at age 46y revealed slow and slightly dysarthric voice, scapula alata, bilateral diffuse weakness (M4- to M5-) with distal and left-sided predominance on both upper limbs, diffuse wasting of the shoulder girdle and upper limb muscles with proximal and left-sided predominance, bilaterally reduced tendon reflexes, hypotonia, a fixed flexion contracture of the left elbow, diffuse weakness (M3 to M5-) with proximal, flexor, and left-sided predominance of the pelvic girdle and lower limb muscles, diffuse wasting with proximal predominance, and reduced freedom of motion of the left ankle joint, reduced tendon reflexes, and hypotonia. The Gower sign was positive.

Routine cardiologic investigation for cardiac involvement at age 26y showed a normal electrocardiogram (ECG) but slightly enlarged left atrium on echocardiography. ECG at age 29y was normal again. Since initiation of steroids at age 39y, she experienced exertional dyspnoea and palpitations, manifesting as sinustachycardia on ECG. Upon administration of beta-blockers at age 41y she developed vertigo and low blood pressure, which is why they were replaced by ivabradine (7.5 mg/d), which was well tolerated and effective.

Table 1. Blood chemical investigations indicating affection of the skeletal muscle

Parameter	RL	10/97	11/00	05/04	05/04	03/07	05/16	08/16
CK	0 - 70 U/L	562	625	2101*	1365*	1968#	371%	nd
GOT	0 - 35 U/L	nd	nd	76	60	58&	nd	26
GPT	0 - 35 U/L	nd	nd	68	61	50&	20	28
LDH	120 - 240 U/L	198	nd	286$	267$	324§	nd	200
Aldolase	0 - 7.6 U/L	7.4	nd	nd	nd	13.9	nd	nd
Myoglobin	0 - 70 µg/L	nd	nd	331	234	nd	nd	nd

CK: creatine-kinase, GOT: glutamate-oxalate transaminase, GPT: glutamate-pyruvate transaminase, *: Reference limit 26-145 U/L, #: reference limit: < 170 U/L, &: reference limit GOT: < 31 U/L, reference limit GPT: < 34 U/L, $: reference limit 135-235 U/L, §: reference limit < 247 U/L, %: reference limit 20-180 U/L, nd: not done.

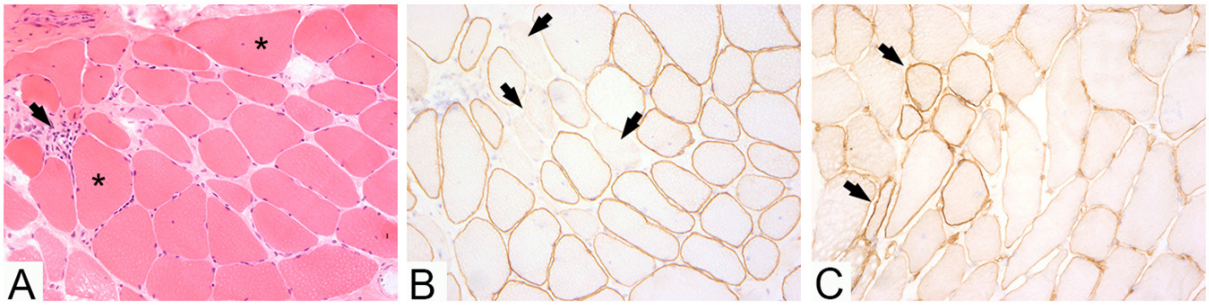

Figure 1. Muscle biopsy from the deltoid muscle shows myopathic features with variation in fibre size and phagocytes invading necrotic fibres. (**A**, H&E; arrow: phagocytes; asterisks: hypertrophic fibres), some fibres lacking dystrophin next to fibres with normal dystrophin expression (mosaic pattern) (**B**, dystrophin; arrows: fibres lacking dystrophin expression), and upregulation of utrophin on the sarcolemma (**C**, utrophin; arrows). ×200

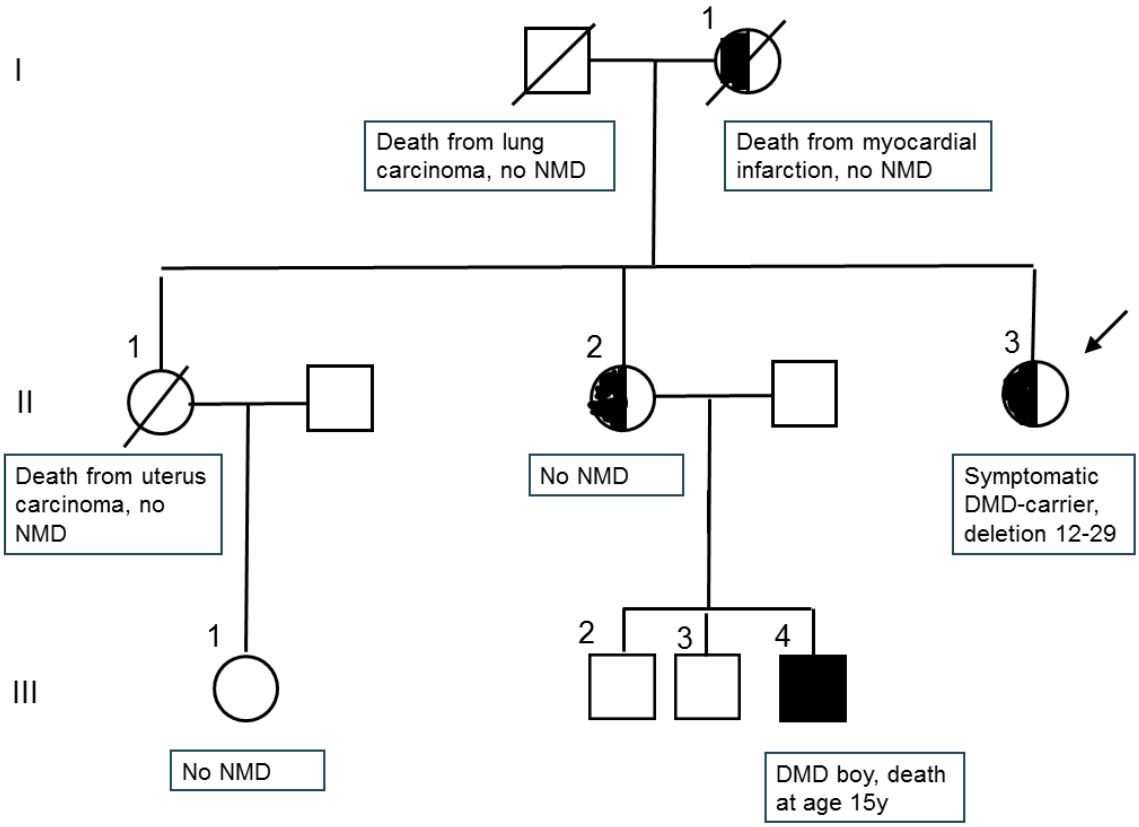

Figure 2. Pedigree of the presented patient. NMD: neuromuscular disorder.

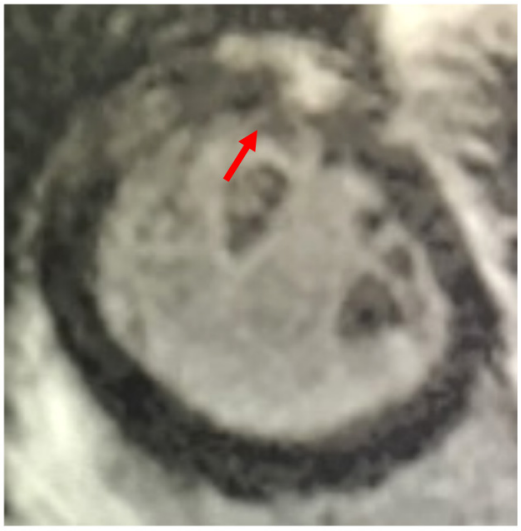

Figure 3. Cardiac MRI (cMRI) at age 41y shows a slightly enlarged left ventricle, mild hypokinesia of the entire myocardium with a left ventricular ejection fraction (EF) of 50%, and focal, transmural late gadolinium enhancement (LGE) of the midventricular lateral wall (arrow).

Cardiac MRI (cMRI) at age 41y revealed mildly reduced systolic function, a slightly enlarged left ventricle, mild hypokinesia of the entire myocardium with a left ventricular ejection fraction (EF) of 50%, and focal, transmural late gadolinium enhancement (LGE) of the midventricular lateral wall (Figure 3). Lisinopril (5 mg/d) was added. At age 45y she consulted a cardiologist because of recurrent chest pain. ECG showed left anterior hemiblock (LAH) and right bundle branch block (RBBB). Echocardiography showed a borderline systolic function (EF 50%), slightly enlarged left atrium, and minimal mitral insufficiency. Since chest pain resolved under non-steroidal analgesics, it was interpreted as vertebrogenic. At follow-up, one month later, she again complained about chest pain and exertional dyspnoea. ECG was unchanged since the previous recording. Echocardiography showed borderline systolic function (EF 50%), mildly enlarged atria, diastolic dysfunction, abnormal mobility of the interventricular septum, and mitral insufficiency. Serum N-terminal pro-brain-natriuretic peptide (NT-pro-BNP) levels were measured several times between age 42y until 46y and were always slightly elevated ranging between 206-244 ng/L (normal < 169 ng/L). Her last medication included lisinopril (2.5 mg/d) and ivabradine (7.5 mg/d).

3. Discussion

DMD-carriers have been previously reported to predominantly manifest phenotypically in the skeletal muscle or myocardium (Table 2) (*2*). Muscle disease in DMD-carriers may have a broad range of manifestations. Muscle disease can be quite variable and may manifest with only creatine-kinase (CK)-elevation (*3,17,22,25,29*), with exercise intolerance (*14*), muscle cramps (*20*), or

Table 2. Phenotypic and genotypic characteristics of published DMD-carriers with or without skeletal muscle involvement and with or without cardiac disease

NOP	Mutation	MM	MB	Myocardium	*Ref.*
15	del (*n* = 10),	Weakness (*n* = 7) CK ↑ (*n* = 15)	Dystrophin ↓ (*n* = 9)	Normal (*n* = 15)	(*14*)
20	del (*n* = 13), dup (*n* = 7)	Symptoms (*n* = 2)	nm	cMRI ab (*n* = 13)	(*8*)
22	nm	nm	nm	LGE (*n* = 7)	(*9*)
1	nm	Weakness	nm	dilation, HF	(*15*)
1	nm	Normal	nm	HF	(*13*)
1	nm	lipid ↑ on MRI	nm	nm	(*16*)
1	nm	CK ↑	nm	ECG, echo an, TnT ↑	(*17*)
5	dup 2	nm	nm	LGE (*n* = 5), HF (*n* = 1)	(*18*)
1	Not detected	Weakness, CK ↑	Dystrophin ↓	LGE	(*19*)
15	del 45-52, 50, 46-49, 51-57, 8-13, dup 43, 17-18, 45-59, PM 14, 15 47, ins 8	Weakness (*n* = 14) Cramps (*n* = 6)	nm	ECG, echo an	(*20*)
7	nm	Weakness (*n* = 5)	nm	LGE (*n* = 4)	(*10*)
1	dup 2	Normal	nm	HF, dystrophin ↓	(*21*)
1	dup 1-6	CK ↑	Dystrophin ↓	HF	(*22*)
38	del 48-50, 3-7, dup 8-9 dup 45, del 6-7	Weakness (*n* = 13) Cramps (*n* = 3)	an (*n* = 2)	dCMP (*n* = 5)	(*23*)
1	ins 43	Weakness	nm	HF	(*24*)
1	nm	CK ↑	nm	Arrhythmias	(*25*)*
1	nm	nm	nm	HF, HTX (26)	
1	nm	Normal	nm	dCMP, dystrophin ↓	(*27*)
1	del 50-52	Normal	Dystrophin ↓	dCMP, HTX	(*28*)
2	nm	CK ↑	nm	Dystrophin ↓, HF	(*29*)
16	nm	CK ↑	nm	ECG, echo an	(*3*)
1	nm	Normal	Dystrophin ↓	dCMP	(*30*)
1	nm	Normal	nm	HF	(*31*)
1	Not detected	Normal	nm	Dystrophin ↓	(*32*)

MM: muscle manifestations, MB: muscle biopsy, LGE: late gadolinium enhancement, HF: heart failure, dCMP: dilated cardiomyopathy, PM: point mutation, nm: not mentioned, an: abnormal, *: during general anesthesia

with muscle weakness and wasting (*14,15,19,20*). Other DMD-carriers may develop wasting without muscle weakness and the more severely affected patients may develop slowly progressive weakness, which may be asymptomatic at onset, as in the presented case. In a study of 16 DMD-carriers, 87% had CK-elevation in the absence of muscle symptoms or signs (*3*). Occasionally, muscle involvement may histologically mimic myositis (*4*). In accordance with these previously described manifestations (Table 2), the currently presented patient manifested with muscle weakness and wasting and CK-elevation.

Concerning cardiac disease in DMD-carriers, frequency, type, degree, and onset can be quite variable. In a study of 210 DMD-carriers 60% had cardiac involvement (*2*). In a Japanese study of 16 DMD-carriers 31% had cardiac symptoms, ECG abnormalities were found in 56%, and 75% had echocardiographic abnormalities (*3*). One of these carriers underwent endomyocardial biopsy showing absence of dystrophin in 75% of the fibers (*3*). Cardiac disease in DMD-carriers includes myocardial fibrosis, ECG abnormalities, systolic dysfunction, or heart failure. Rarely, DMD-carriers may develop dilated cardiomyopathy (Table 2) (*5,6*), even as the initial cardiac manifestation (*5*). In a single DMD-carrier sudden cardiac death was reported (*7*). In a study of 20 DMD-carriers of whom only 2 had muscle symptoms, 13 had at least one cMRI abnormality, 4 (20%) had reduced systolic function, and 13 (65%) had LGE (*8*). All LGE-positive patients had non-ischemic LGE with subepicardial involvement of the lateral free wall being the most frequent pattern (*8*). In another study of 20 DMD-carriers one third had LGE (*9*). Muscle and cardiac symptoms were not different between those with and without LGE (*9*). In a cMRI study of 7 DMD-carriers, LGE was found in four of them (*10*). LGE was subendocardial but two patients had focal LGE in the left inferolateral wall (*10*). In the majority of the cases, cardiac involvement in DMD-carriers does not develop before adulthood. In a study of 23 DMD-carriers under age 16y, none had any abnormality on extensive cardiologic work-up (*11*). However, in some patients cardiac disease may already develop in childhood. In a 10yo DMD-carrier, systolic dysfunction was recognised already at age 10y in the absence of muscle manifestations (*12*). Frequently, cardiac involvement may precede muscular abnormalities (*3,10,13*). Cardiac dysfunction in DMD-carriers may be triggered by pregnancy (*13*) or steroids, as shown in our case. In accordance with previous reports (Table 2), the currently presented patient manifested cardiologically with heart failure, reduced systolic function, LGE, and ECG abnormalities.

Whether the degree of muscle and cardiac involvement

only depends on the degree of X-chromosome inactivation or additionally depends on the location and size of the dystrophin deletion has been only poorly investigated. Exons most frequently deleted in DMD-carriers include numbers > 45 (Table 2). Though there have been reports according to which deletions in the N-terminal domains are predominantly associated with cardiac involvement, these reports have not been confirmed in later studies. From Table 2 it is not possible to draw a strong correlation between the location of the mutation within the dystrophin gene and the type and degree of muscle or cardiac involvement.

In conclusion, this case shows that muscle manifestations in DMD-carriers may start years before cardiac involvement becomes apparent. Muscle and cardiac manifestations may slowly progress over years. Systolic dysfunction, heart failure, and conduction defects are attributable to myocardial fibrosis, manifesting as LGE. Application of steroids may trigger cardiac involvement, and this is why they should be given with caution in DMD-carriers.

Note: Informed consent: Informed consent was obtained from the described participant

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(Received January 6, 2018; Revised February 25, 2018; Accepted March 21, 2018)

Case Report

DOI: 10.5582/irdr.2018.01022

Progressive disseminated histoplasmosis in an immunocompetent adult: A case report

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Summary **Histoplasmosis is a systemic fungal infection caused by *Histoplasma capsulatum* which occurs endemically in some parts of the world like North and Central America particularly in Mississippi and Ohio River valleys, but is uncommon in India. Progressive disseminated form of histoplasmosis (PDH) usually occurs in the immune-compromised hosts especially in HIV positive population. In PDH any organ can be involved like lung, liver, spleen, brain, adrenals *etc*. Involvement of oral cavity and buccal mucosa in PDH is common but pharyngeal involvement is rare. We here report a case of progressive disseminated histoplasmsosis with pharyngeal involvement in an immunocompetent male from non-endemic area. This case presented to us with history of long duration fever and we found the etiology by giving due significance to a trivial symptom and thorough evaluation of the same. Etiology was found as disseminated histoplasmosis, which is not a common disease. We treated him initially with amphotericin-B then subsequently with itraconazole for one year. He recovered fully over the period of one year with the given treatment. This case report emphasizes that disseminated histoplasmosis should be considered one differential diagnosis in case of long duration of fever, even in an immunocompetent patient. It also emphasizes that in evaluation of a case of long duration of fever, even a trivial symptom is very crucial, which may direct towards the diagnosis.**

Keywords: Progressive disseminated histoplasmosis, immunocompetent, pharyngeal histoplasmosis, *Histoplasma capsulatum*, fever of unknown origin

1. Introduction

Histoplasmosis is a systemic fungal infection caused by *Histoplasma capsulatum* which occurs endemically in some parts of the world, but is uncommon in India (*1*). *Histoplasma capsulatum* is endemic in many parts of the world like North and Central America particularly in Mississippi and Ohio River valleys. Travel to these endemic regions is an important predisposing factor for occurrence of histoplasmosis in individuals belonging to non-endemic areas (*1*). Soil contaminated with bird droppings acts as the main environmental reservoir for this fungi. It exists in the environment in mold form as well as in forms of microconidia and macroconidia, while in tissue it exists as yeast form. The microconidia are the infectious form and inhalation of these is the major route of infection. Involvement of individuals in activities that disturb the soil and bird droppings like excavation and construction activities in the endemic regions expose individuals to the microconidia of the fungi (*1*). Progressive disseminated form of histoplasmosis (PDH) usually occurs in the immune-compromised hosts especially in the HIV positive population (*2*). Involvement of oral cavity and buccal mucosa in PDH is common but pharyngeal involvement is rare.

We here report a case of progressive disseminated histoplasmsosis with pharyngeal involvement in an immunocompetent male from non-endemic area.

Released online in J-STAGE as advance publication May 15, 2018.

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2. Case Report

A 54 year old, previously healthy male from Agra,

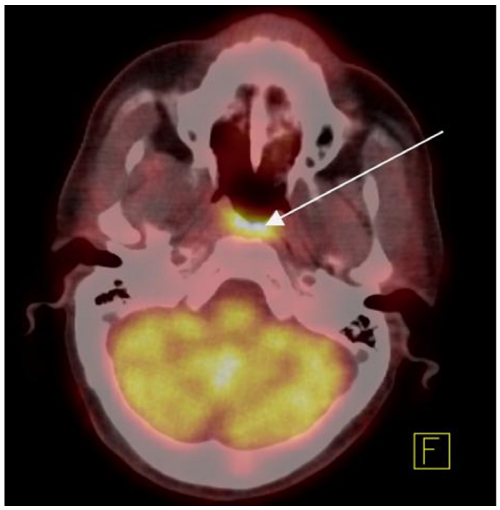

Figure 1. FDG-PET scan showing significantly increased FDG uptake in the posterior pharyngeal wall.

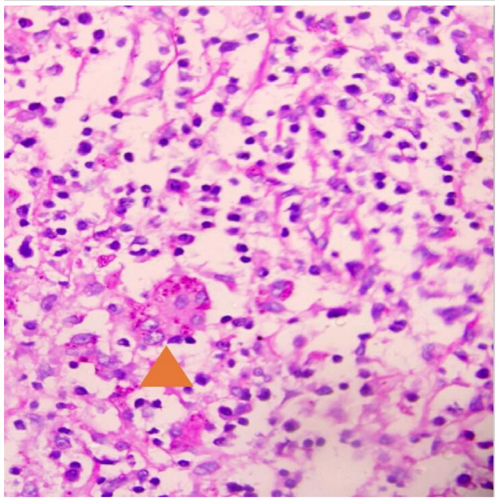

Figure 2. Yeasts of histoplasma are seen extracellularly as well as inside the giant cells under 40× magnification in the biopsy specimen taken from pharynx (Periodic acid Schiff staining with diastase).

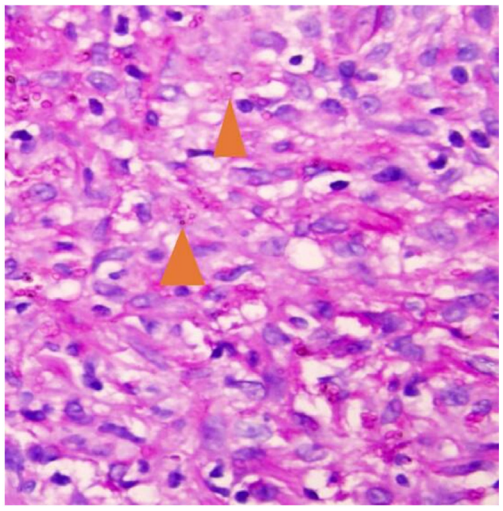

Figure 3. Yeast form of histoplasma is seen under 40× magnification in the biopsy specimen taken from pharynx (Periodic acid Schiff staining with diastase).

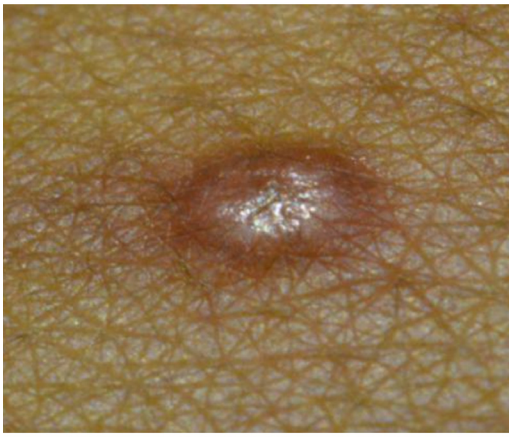

Figure 4. Nodular skin lesion over chest developed after starting amphotericin B.

Northern India was admitted to us with complaints of intermittent and moderate to high-grade fever of four months duration. Fever was associated with mild dry cough, night sweats, anorexia and weight loss of about five kg over four months. There were no musculoskeletal, abdominal or genitourinary symptoms. He was not a diabetic and had been treated for pulmonary tuberculosis 30 years ago. One month prior to presentation to us, he was prescribed empirical anti tubercular therapy, which he took for around 3 weeks without any relief and stopped by himself.

His general physical examination was unremarkable, except for presence of mild pallor. Systemic examination revealed mild hepatosplenomegaly. Laboratory parameters showed mild anaemia (Hb – 10.1 gm/dL) with normal ESR. His blood glucose was in normal range, liver and kidney function tests were also normal with normal albumin to globulin ratio. Investigations for malaria, kala-azar, brucellosis, HIV and autoimmune markers were negative. Bone marrow examination showed reactive changes with no evidence of Leishmania Donovani (LD) bodies, granulomas and abnormal cells. Contrast enhanced CT scan of thorax and abdomen revealed hepato-splenomegaly with no focal lesions and echocardiography showed no vegetations. During hospitalisation patient continued to have intermittent spikes of moderate grade fever. On further evaluation, his FDG Positron Emission Tomography (PET) scan (Figure 1) showed uptake in the region of posterior pharyngeal wall which was reported as likely to be physiological. Around the same time, patient developed mild throat discomfort and pain during swallowing. His throat examination showed mild congestion on posterior pharyngeal wall and soft palate. He received symptomatic treatment with decongestants but his symptoms did not improve and subsequently ENT consultation was sought. On endoscopic examination, he was found to have unhealthy mucosa of posterior

pharyngeal wall and soft palate with multiple whitish ulcers. Multiple biopsies were taken from these ulcers. Histopathological examination of these biopsy specimens was done initially with hematoxylin and eosin stain followed by Gomori methenamine silver and Periodic acid Schiffs with diastase (PAS-D) stain, which revealed multiple, small yeast like fungus both intra and extracellularly, highly suggestive of *Histoplasma capsulatum* (Figures 2 and 3). Later on, the culture from the same specimen grew *Histoplasma capsulatum*.

Patient was started on liposomal amphotericin B (3 mg/kg/day) after which the fever and throat symptoms disappeared within a week. After three days since starting the treatment, patient developed pruritic papulo-nodular rash over his neck and chest (Figure 4). Biopsy from these skin lesions was also suggestive of *Histoplasma capsulatum*. Patient responded well to liposomal amphotericin B and after two weeks was switched to oral itraconazole therapy (200 mg BD). Patient tolerated the drugs well and continued to improve over the next few days. After six months of treatment, he continued to be afebrile and gained weight. He was given oral itraconazole for one year, and he recovered fully; subsequently his medication was stopped.

3. Discussion

Histoplasmosis is a systemic fungal infection caused by dimorphic fungi *Histoplasma Capsulatum* (*1*). Activities that disturb the soil and bird droppings like excavation and construction activities in the endemic regions expose individuals to the spores (micro-conidia) of the fungi (*1*). Only less than one percent of exposed individuals develop disease and the development of the symptoms depends on the level of exposure as well as the immune status of the person exposed (*2*). Histoplasmosis can be clinically classified into pulmonary histoplasmosis and extrapulmonary or disseminated histoplasmosis. Acute pulmonary histoplasmosis occurs after initial infection with this fungus, mostly in children. It is usually a self-limited illness which mimics viral respiratory tract infections with fever, malaise, arthralgias, cough, and chest pain. Chronic cavitatory form of pulmonary histoplasmosis occurs mainly in individuals with pre-existing lung diseases especially emphysematous lungs. The presentation is very similar to pulmonary tuberculosis as patients usually have fever, malaise, anorexia and weight loss with cough, expectoration, dyspnoea and haemoptysis and the chest X ray shows large cavitatory lesions and fibrosis (*1*). In individuals with compromised immunity (diabetes, HIV positive patients with CD4 < 150, alcoholics and those on immunosuppressive drugs) the fungus disseminates to various sites causing the life threatening form of disease called PDH (*1*). The involvement of two or more sites by the fungus defines the diagnosis of disseminated histoplasmosis, which in our case was seen as involvement of oropharynx and skin. Disseminated form of histoplasmosis can present as acute, subacute or chronic PDH (*1*). PDH is uncommon in India and until now less than 200 cases have been reported. Most of these cases belong to the Eastern and North-Eastern states of India, mostly from West Bengal and Assam (*2-5*). Our patient belonged to Agra (Northern India) from where PDH has been reported very rarely. It is very likely that many cases remain undiagnosed due to non-availability of specific diagnostic tests or misdiagnosed as tuberculosis which is so rampant in India. The under reporting of cases is also a reason behind the meager data available in India for histoplasmosis.

Nearly 75-80% cases of PDH present as fever of unknown origin with constitutional symptoms, anorexia and weight loss (*3-5*). This fungus can disseminate from lungs to virtually every organ of the body and commonly spreads to liver, spleen, adrenal glands, lymph nodes, bone marrow and gastro intestinal tract. Hepatosplenomegaly is found in nearly 50 % of patients (*3-5*) and gives an early clue towards dissemination of the disease. Respiratory symptoms and lymphadenopathy is also common. Since clinically histoplasmosis mimics disseminated tuberculosis, it is not uncommon for these patients to receive empirical anti-tubercular therapy before being diagnosed as histoplasmosis, thus delaying the diagnosis (*3*).

Oropharyngeal involvement is seen in 30-40% of cases of PDH and most commonly involves buccal mucosa (54.8%) followed by tongue and palate (*6*). Involvement of pharynx and larynx is rare. Lesions may notoriously mimic malignancy both clinically and pathologically and pose a great challenge for the clinicians in diagnosis and management (*7*). Many patients having oropharyngeal disease also have simultaneous involvement of adrenal glands which can present as life threatening adrenal insufficiency.

Diagnosis of PDH relies mainly on the histopathology of the biopsy from affected tissues with a sensitivity of 75-80% (*8*). Culture remains the gold standard for diagnosis of histoplasmosis with sensitivity of 74% with highest yield from bone marrow and blood (*8*). Measurement of histoplasma antigen in serum and urine is a very useful test as it has high sensitivity of 94% and it can be used to monitor the response to therapy (*8*).

Treatment of PDH involves administration of liposomal amphotericin B (3-5 mg/kg/day) for 2 weeks followed by a maintenance therapy with oral itraconazole 200 mg BD for at least one year with regular monitoring of histoplasma antigen level in serum or urine (*8*). We were not able to monitor histoplasma antigen levels due to non-availability of this test at our institute.

Thus PDH though uncommon, is not rare in India and needs to be considered as a differential diagnosis in a case of fever of unknown origin even in

immunocompetent adults belonging to non-endemic regions. Although individually both the throat findings as well as the findings in the FDG PET scan were subtle, correlating them and subjecting the patient to further evaluation proved vital. This highlights the fact that in a case of fever of unknown origin subtle symptoms and signs should not be considered insignificant and should be evaluated thoroughly, as these could be potential diagnostic clues (PDCs). However, absent initially these PDCs could appear any time during the course of illness and should be pursued further.

Acknowledgements

We would like to thank Dr. Alok Thakkar (Professor, Dept of ENT AIIMS New Delhi) for his precious clinical inputs in diagnosing the case and for providing images of throat examination. We would also like to thank Dr. M C Sharma (Professor Dept of Pathology AIIMS New Delhi) for helping in diagnosis and providing images of pathological examination of specimens.

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(Received February 24, 2018; Revised May 1, 2018; Accepted May 6, 2018)

Case Report

DOI: 10.5582/irdr.2018.01031

Breast abscess due to *Salmonella paratyphi* A : Case reports with review of literature

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Summary ***Salmonella paratyphi* A causes paratyphoid fever which is characterized by acute onset of fever, abdominal pain, diarrhoea, nausea and vomiting. Localized disease can occur following both overt and silent bacteremia followed by seeding of bacteria at distant sites. *Salmonella* species though associated with abscess formation in various organs,are rarely associated with breast abscess. We report 2 cases of breast abscess due to *Salmonella enterica* serotype paratyphi A. Appropriate sampling, surgery supplemented by a comprehensive microbiological work up aided in pathogen identification and appropriate antibiotic administration for a successful outcome of these patients.**

Keywords: Breast absecess, *Salmonella* species, haematogenous spread, pus, drainage

1. Introduction

Salmonellae are gram-negative bacilli and disease caused by *Salmonella* organisms can be divided into 2 categories: typhoidal and non-typhoidal. The reservoir for typhoidal disease is human, but non-typhoidal salmonellae are widely distributed among animals. In humans, nontyphoidal Salmonella infections are most often associated with food products; the rest is nosocomial infections or is acquired from pets (*1*). The aetiological agents of breast abscess are diverse, *Staphylococcus aureus* is the most common cause of breast abscess (*2*) (Table 1). *Salmonella* species though associated with abscess formation in various organs,are rarely associated with breast abscess. We report 2 cases of breast abscess due to *salmonella enterica* serotype paratyphi A. Drainage of the abscess followed by appropriate antibiotic treatment resulted in a good clinical outcome. We also reviewed the literature on this entity.

2. Case Reports

Released online in J-STAGE as advance publication May 23, 2018.

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2.1. *Case I*

A 27-year-old female presented to the surgical outpatient department of AIIMS hospital in 2017 with complaints of painful lump in her left breast for 2 months duration. The patient's history revealed that the lump had started to grow in size gradually for two months. The lump was initially firm and painless, but gradually grew in size and became painful. The patient was not pregnant or lactating at that time. The patient had no other co-morbidities. She was afebrile on admission, and all the vital signs were within normal limits. On local examination, the left breast was tender and swollen. A mobile, soft, fluctuating mass of around 5 × 6 cm in size located in the left lower quadrant was palpable. The overlying skin was warm and erythematous. There was no spontaneous discharge from the abscess and no lymphadenopathy. There was no nipple retraction or discharge from the nipple. Axillary lymph nodes were not palpable. Rest of physical and systemic examination was normal. Ultrasonography (USG) of the left breast revealed a heterogeneously hypoechoic deep-seated irregular collection of approximately 4.9 × 3.5 cm size. A diagnosis of breast abscess was made. The abscess was drained by USG guided aspiration and treated empirically with oral amoxicillin-clavulanic acid 625 mg 8 hourly. Her laboratory parameter revealed a total leukocyte count of 12,500/mm^3, with 70% polymorphonuclear leukocytes. Pus sample was

Table 1. Causative Organisms for Breast Abscesses (*2*)

Aerobic gram-positive bacteria
Staphylococci, Streptococci
Aerobic gram-negative bacteria
Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, Salmonella spp
Anaerobic bacteria
Peptostreptococcus, Propionibacterium, Bacteroides, Lactobacillus, Eubacterium, Clostridium,Fusobacterium, Veillonella
Fungi
Candida, Cryptococcus
Other causes
Bartonella henselae, Mycobacteria, Actinomyces, Brucella
Parasites
Maggot infestation

collected and sent for microbiological investigations. Ziehl-Neelsen stain did not demonstrate acid fast bacilli (AFB) and GeneXpert was negative for *Mycobacterium tuberculosis*. Gram stain of pus showed numerous polymorphonuclear leukocytes and Gram-negative bacilli. Pus culture grew cream coloured non-hemolytic colonieson 5% sheep blood agar and nonlactose-fermenting colonies on MacConkey agar which were identified as *Salmonella enterica* paratyphi A by matrix-assisted laser desorption ionization-time of flight mass spectrometry using the bioMérieux VITEK MS system (IVD database version 2.0) (USA). The antibiotic susceptibility was determined by Kirby Bauer disk diffusion method in accordance with CLSI guidelines and the isolate was susceptible to ciprofloxacin, trimethoprim-sulfamethoxazole, erythromycin, azithromycin, chloramphenicol, ceftazidime, ceftriaxone, ampicillin and Nalidixic acid.

The patient was called back to re-evaluate the detailed history. Three months ago she had gastroenteritis which resolved without antimicrobial treatment. Stool, urine, and blood samples were obtained for culture and found negative for *Salmonella paratyphi* A spp. Widal test was also found negative. Subsequently she was treated with tablet azithromycin 1000 mg once daily for five days. The patient responded well to the treatment. The patient was cured after radiological aspiration of the abscess and antibiotic therapy. The abscess did not recur after one year of follow-up.

2.2. *Case 2*

A 29-year-old diabetic female presented to the surgical outpatient department of our hospital with complaints of a lump in her left breast of past 20 days duration in 2017. The patient was not pregnant or lactating at that time. Local examination revealed a lump 4.5 × 3.5 cm in left breast at 2 o'clock position free from skin and underlying muscle. The overlying skin appeared normal, with no inflammation and was non tender. There was no nipple retraction or discharge from the nipple. Axillary lymph nodes were not palpable. Rest of general physical and systemic examination was normal. Provisional diagnosis of breast lump with a suspicion of malignancy was made and following investigations were advised: routine blood investigation, Chest x-ray, USG of both breasts, and core needle biopsy of the left breast lump. Her routine blood investigations were normal. USG of left breast revealed heterogeneously hypoechoic mass lesion of approximately 3.4 × 2.4 cm size in the left breast at 2 o'clock position. Core needle biopsy finding showed plenty of neutrophils and inflammatory cells with necrotic background suggestive of breast abscess. Lump excision with radical duct excision was done under sterile conditions. The patient was empirically started on tablet erythromycin 500 mg 6 hourly and metronidazole 400 mg BD for two weeks. The drained pus was collected and sent for microbiological investigations. Gram stain of the drained pus revealed gram-negative bacilli. The culture on blood agar grew grey-white opaque, non-hemolytic colonies and non lactose fermenting colonies on MacConkey's agar. This non-lactose fermentating gram negative bacillus was identified as *Salmonella paratyphi* A by MALDI-TOF mass spectrometry using the bioMérieux VITEK MS system (IVD database version 2.0). The antibiotic susceptibility was determined by Kirby Bauer disk diffusion method in accordance with CLSI guidelines and the isolate was susceptible to ciprofloxacin, trimethoprim-sulfamethoxazole, erythromycin, azithromycin, chloramphenicol, ceftazidime, and ceftriaxone ampicillin and nalidixic acid. On receipt of the pus culture report blood culture and widal test were advised. However, both these tests were negative. She was treated with oral ciprofloxacin 500 mg twice daily for 14 days. USG of breast done after two weeks of antibiotic therapy revealed no significant change in the heterogenoushypoechoic lesion. Left breast lumpectomy with left radical milk duct excision was done. Some pus like material was encountered which was sent to microbiology laboratory for culture and

Table 2. Showing the reports of breast abscess cases due to *Salmonella Typhi* and *Paratyphi* infections

Year	Age of Patient	Underlying condition	*Salmonella* Species	Unilateral/bilateral breast abscess	Clinical presentation	References
2016	60	Diabetes mellitus	*S. Typhi*	Unilateral	Acute	Murugesan *et al*.(*10*)
2016	unknown	Unknown	*S. Typhi*	Unknown	Unknown	Elumalai *et al*. (*11*)
2015	37	Diabetes mellitus	*S. paratyphi* A	Unilateral	Acute	Sood (*5*)
2014	31	Unknown	*S. paratyphi* A	Unilateral	Recurrent	Ghadage *et al*. (*6*)
2013	40	Unknown	*S. Typhi*	Unilateral	Acute	Banu *et al*. (*12*)
2012	33	Unknown	*S. paratyphi* A	Unilateral	Chronic	Siddesh *et al*. (*7*)
2012	60	Diabetes mellitus	*S. Typhi*	Unilateral	Acute	Kumar *et al*. (*13*)
2012	33	Unknown	*S. paratyphi* A	Unilateral	Recurrent	Fernando *et al*. (*8*)
2011	28	Unknown	*S. Typhi*	Unilateral	Acute	Vattipally *et al*. (*14*)
2011	29	Unknown	*S. Typhi*	Bilateral	Acute	Singh *et al*. (*15*)
2009	35	Unknown	*S. Typhi*	Bilateral	Acute	Singh *et al*. (*16*)
2007	Unknown	Immunocompromised	*S. Typhi*	Unilatearal	Acute	Mahajan *et al*. (*17*)
2007	54	Unknown	*S. Typhi*	Unilatearal	Acute	Delori *et al*. (*18*)
2003	40	Fibroadenoma of breast	*S. Typhi*	Unilateral	Acute	Jayakumar *et al*. (*19*)
1994	Unknown	Unknown	*S. Typhi*	Unknown	Unknown	Lalitha *et al*. (*20*)
1972	43	Unknown	*S. Typhi*	Unilateral	Chronic	Barrett *et al*. (*21*)
1907	16	Unknown	*S. Typhi*	Unilateral	Chronic	Thayer *et al*. (*22*)

Table 3. Showing the reports of breast abscess cases due to *Salmonella* Paratyphi infections (*5-8*)

Year	Underlying condition	Unilateral/bilateral breast abscess	Clinical presentation	Treatment
2015	Diabetes mellitus	Unilateral	Acute	Injectable Ceftriaxone/Duration unknown
2014	Unknown	Unilateral	Recurrent	Injectable cefotaxime/Duration unknown
2012	Unknown	Unilateral	Chronic	Injectable Ceftriaxone for 2 weeks
2012	Unknown	Unilateral	Recurrent	Injectable Ceftriaxone for 6 weeks
Case 1	Gastroenteritis	Unilateral	Acute	Oral azithromycin for five days
Case 2	Diabetes mellitus	Unilateral	Acute	Intravenous ceftriaxone for seven days

sensitivity. Culture again grew *Salmonella paratyphi* A with similar antimicrobial susceptibility pattern. According to the susceptibility pattern of the strain, the antibiotic regimen was rationalized intravenous ceftriaxone 2 gm 12 hourly for seven days. On follow-up there was complete resolution of abscess.

3. Discussion

Salmonella paratyphi A causes paratyphoid fever which is characterized by acute onset of fever, abdominal pain, diarrhoea, nausea and vomiting (*3,4*). Localized disease can occur following both overt and silent bacteremia followed by seeding of bacteria at distant sites (*3*). Breast abscess due to *Salmonella paratyphi* is rare complication of enteric fever. It can be attributed mainly to *Salmonella typhi* (Table 2). Till date, there are only four reports of breast abscess caused by *Salmonella paratyphi* A (Table 3) (*5-8*). The first case of breast abscess due to *Salmonella paratyphi* A was reported in 2012 by Fernando *et al*. in a young woman from Bangladesh (*8*).

We could not determine the source of *Salmonella Paratyphi* A in both cases, however our first patient gave a history of gastro-enteritis. In enteric fever, dissemination to multiple organ systems following bacteremia may lead to localized abscess formation. Although hematogenous dissemination of *Salmonella* is a well established and the most likely mechanism of breast abscess, in the second case, this route of dissemination seemed unlikely. However, since our patient is diabetic, we speculate that impairment of cell-mediated immunity in diabetes may have impaired the ability of macrophages to kill intracellular pathogens such as *Salmonella*. Underlying condition was unknown in previously reported *Salmonella Paratyphi* A cases (*5-8*). Our both cases had unilateral breast abscess like other reported cases (*5-8*).

There are cases of recurrent breast abscess caused by *Salmonella Paratyphi* A reported in literature (*6,8*). However, unlike all these cases of *Salmonella Paratyphi* A breast abscess in our patient did not have any recurrence or chronicity of abscess. Kumar reported a multidrug resistant typhoid with breast abscess (*9*). In the present case, the *Salmonella Paratyphi* A isolate showed good susceptibility to all the drugs. However, blood culture and widal test results were negative in these patients. But the pus from breast aspirated under sonographic guidance grew the same isolate as pure growth implicating *Salmonella Paratyphi* A as the causative agent of breast abscess in this patient. The histological examination was also suggestive of breast abscess.

Due to its diverse aetiologies, it can pose a diagnostic challenge and warrants detailed evaluation. As observed in both our cases, the importance of identifying atypical

agents is that appropriate antimicrobial therapy can be instituted early.

Several line of evidence suggest that *Salmonella Paratyphi* A isolated from the patients was responsible for the breast abscess: gram stain of aspirated pus was positive for gram negative bacteria, culture of aspirated pus grew *Salmonella Paratyphi* A, direct evidence of infection was present, there was an absence of other pathogen and infection responded to treatment.

4. Conclusion

Appropriate sampling, surgery supplemented by a comprehensive microbiological work up aided in pathogen identification and appropriate antibiotic administration for a successful outcome of these patients.

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(Received April 4, 2018; Revised May 14, 2018; Accepted May 16, 2018)

Case Report

DOI: 10.5582/irdr.2018.01034

New onset hyperglycemia attributed to renal cell carcinoma

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Summary **A 61-year-old male was admitted from the outpatient setting for treatment of severe hyperglycemia. Five months earlier, his hemoglobin A1c had been 5 mmol/mol. At presentation, hemoglobin A1c was 11.3 mmol/mol and he required insulin therapy at discharge. Later magnetic resonance imaging (MRI) identified bilateral renal masses, previously seen on ultrasound during workup for chronic kidney disease, as being suspicious for renal cell carcinoma (RCC). He underwent partial nephrectomy and cryoablation with pathology showing papillary type RCC. Hyperglycemia resolved after resection and insulin therapy was discontinued, requiring only an oral hypoglycemic. Hyperglycemia as a paraneoplastic syndrome related to RCC is rare. The cause of this acute hyperglycemia is not understood, though previously suggested mechanisms include ectopic glucagon production, autoimmune causes and interleukin-6 (IL-6) mediated pathways. Severe, new-onset hyperglycemia in the absence of common causes and with a renal mass on imaging may represent an uncommon paraneoplastic syndrome secondary to RCC.**

Keywords: Hyperglycemia, malignancy, paraneoplastic syndrome, renal cell carcinoma

1. Introduction

Renal cell carcinoma (RCC) has an incidence of approximately 65,000 cases annually in the United States with approximately 15,000 deaths a year (*1*). Over time, mortality rates have been steadily decreasing along with decreasing tumor size at the time of initial finding, likely due to an increased rate of incidental detection on abdominal imaging (*2*). Currently, most RCC is diagnosed while the disease is still localized, with 65% of cases diagnosed while still confined to the kidney. While earlier stages of RCC have a 5-year survival of approximately 90%, stages III and IV have a 5-year survival rate of 59-70% and median survival of 1 year respectively. It is estimated that approximately 20% of patients with RCC will have manifestations of paraneoplastic syndromes and in a significant number of patients; this may be the presenting complaint leading to a diagnostic workup and detection of RCC (*3*). The most common paraneoplastic syndromes associated with RCC are anemia, erythrocytosis, thrombocytosis, fever, cachexia, AA amyloidosis, hepatic abnormalities (Staufer's syndrome), and polymyalgia rheumatica. Only a handful of cases have reported hyperglycemia secondary to RCC paraneoplastic syndrome with one case reported in the past decade. Here we describe the case of a patient whose presentation with severe hyperglycemia without previous history of diabetes, contributes to the diagnosis of renal cell carcinoma.

2. Case Report

A 61-year-old male with a history of essential hypertension, hyperlipidemia, coronary artery disease with past stenting and chronic kidney disease (CKD) was sent for admission from his nephrologist's office in October of 2016 when found to have serum blood glucose of 667 mg/dL. Per hospital records, the patient had not had a hemoglobin A1c over 5.5 mmol/mol since the beginning of his records 8 years ago with no diabetes medication during that time. A month prior to admission, the patient was started on metformin 500 mg twice daily by another provider and he reported he had run out of medication 10 days ago. At presentation,

Released online in J-STAGE as advance publication May 25, 2018.

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he complained of fatigue, polyuria, polydipsia, blurred vision and lightheadedness. Further review revealed the patient had a renal ultrasound a month before this admission while establishing care with a nephrologist for management of his CKD. At that time, bilateral renal masses included a 3.8 × 2.9 × 3.7 cm hypo echoic, exophytic mass on the right kidney and 1.6 cm 1.3 × 1.4 cm hypo echoic solid mass on the left kidney. Suggestion for urologic consultation and further imaging to rule out malignancy had been made, though these had not yet been completed.

On physical exam, the patient was afebrile with heart rate of 89 beats per minute and blood pressure of 104/74 mmHg and saturating at 96% on room air. He was not in distress, oral mucosa was dry and he was alert, awake and oriented. Lab values were significant for sodium of 129 mEq/L, potassium 4.8 mEq/L, bicarbonate of 22 mEq/L, blood urea nitrogen of 36 mEq/L, serum creatinine of 2.3 mEq/L and serum glucose of 667 mg/dL. Calculated serum osmolality was 308 mOsm/kg, liver panel was normal, white blood cell count of 6.3 × 10^3 cells/µL and hemoglobin of 11.3 × 10^6 cells/µL. Serum pH was 7.39 with no detectible beta-hydroxybutyrate and hemoglobin A1C was 11.3 mmol/mol and otherwise ranging from 4.5-5.5 mmol/mol over the last eight years.

During admission, computed tomography (CT) abdomen displayed a right indeterminate 3.6 × 3.7 cm exophytic inferior cortical renal mass. Additionally, there was a 1.0 × 0.7 cm left mid-pole lesion. Upon urologic evaluation, the CT scan findings were thought to be low risk for malignancy and reimaging in 6 months was suggested. The patient was discharged on insulin, requiring up to 43 units per day, with instruction to follow up with an ultrasound of the abdomen. Repeat ultrasound six months later revealed an enlarging right renal mass measured as 4.2 × 4.0 cm in diameter and left to 1.3 cm and patient was scheduled for MRI to further characterize the mass. MRI re-demonstrated the right sided mass that had increased to 4.5 cm with diffuse post contrast enhancement (Figure 1) as well as left sided hypo-intense mass measuring 1.2 cm with post contrast enhancement (Figure 2) concerning for renal cell carcinoma. The patient underwent right-sided partial nephrectomy with pathology revealing papillary renal cell carcinoma, type 1, and grade 2. Immunohistochemical stains showed tumor cells positive for CK7 and vimentin and negative for CD117, consistent with a diagnosis of RCC (Figure 3). Left sided renal mass was treated with cryoablation and interventional radiologic (IR) embolization. The patient recovered well from his surgery and was discharged.

Four months after discharge, patient's hemoglobin A1c had trended down to 4.5 mmol/mol and he was switched from insulin to glipizide 2.5 mg. The patient was contacted after discharge and informed consent was obtained for publication of this study.

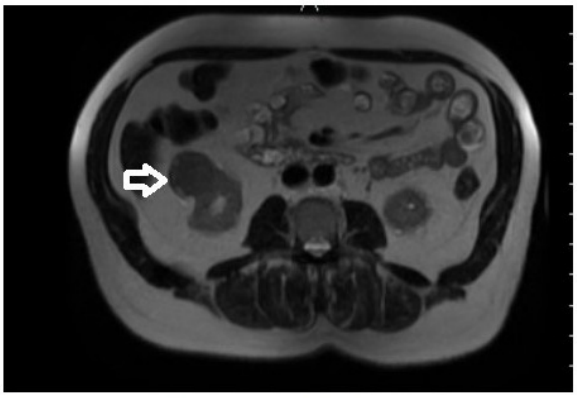

Figure 1. MRI of the abdomen displaying round exophytic mass of the right kidney increased to 4.5 cm from 4 cm a month prior.

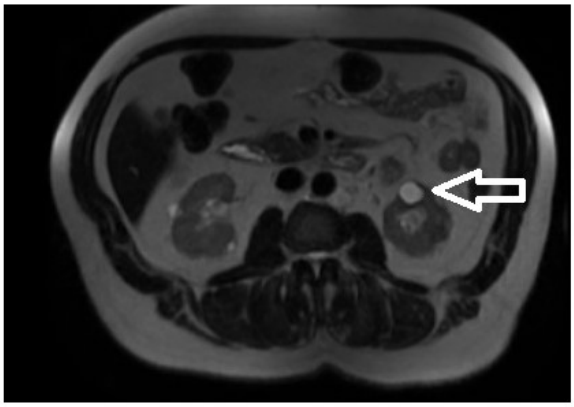

Figure 2. MRI of the abdomen displaying left sided, renal, hypo-intense mass measuring 1.2 cm with post contrast enhancement.

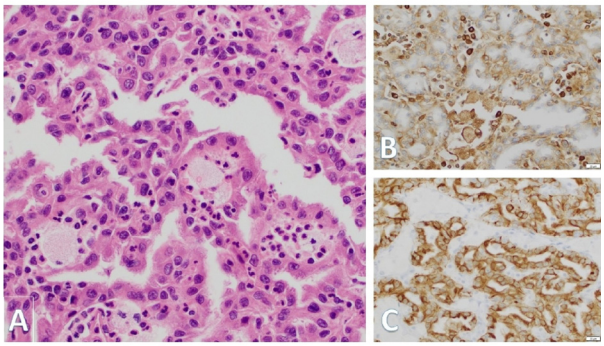

Figure 3. Histopathology of right renal mass following nephrectomy. (A) Hematoxylin and eosin stain; **(B)** Immunohistochemical staining positive for CK7 and for **(C)** Vimentin. The findings are consistent with a diagnosis of renal cell carcinoma.

3. Discussion

In this case, a patient with no former history of diabetes and with a hemoglobin A1c of 5.5 mmol/mol just five months prior to this hospitalization presented with severe hyperglycemia. During this five-month

Table 1. Review of previously reported cases

Author	Year	Synopsis
Pavelic (*6*)	1981	59 yo F, no history given with left flank pain. Left kidney mass 25 × 15 × 8 cm. Detected high levels of serum glucagon and insulin, which trend down after surgery, fluctuating blood sugars.
Palgon (*8*)	1986	67 yo F with no previous IDDM, presents with AMS and serum glucose 650 mg/dL and requires 50 U insulin per day. Eight cm diameter mass, no metastasis, clear cell type, glucagon and somatostatin stains negative. Right radical nephrectomy with no insulin required after resection.
Jobe (*10*)	1993	66 yo M, with no previous IDDM, presents in DKA with serum glucose of 847 mg/dL and requires up to 80 U/day of insulin. Right renal mass 9 × 10 × 11.5 cm, no metastasis, and clear cell type. Right nephrectomy with no insulin required after removal. Discharged on 2.5 mg of glyburide.
Callewaert (*4*)	1999	35 yo F with previous IDDM requires 600 U/day of insulin at presentation. Bilateral multifocal papillary renal cell carcinoma, grade 2, with no metastasis. Bilateral radical nephrectomy and returns to premorbid insulin use of 80 U/day.
Macaulay (*7*)	2002	69 yo M with previous non-IDDM (on gliclazide) with hyperglycemia and elevated alkaline phosphatase requiring insulin (amount not specified). Six cm, right, necrotic mass, grade 2 with renal vein involvement, no metastasis. Left radical nephrectomy with return to gliclazide after nephrectomy.
Elias (*5*)	2005	52 yo F with no previous IDDM admitted with DKA. Elevated anti-GAD and islet cell antibody, which normalized after nephrectomy. Negative stain for glucagon, growth hormone and insulin. Insulin amounts prior and following tumor removal not specified though noted as decreasing.
Harada (*9*)	2016	68 yo F with no previous IDDM, presents with serum glucose 353 mg/dL and A1c of 11.7 mmol/mol, elevated alkaline phosphatase requires 43 U/day insulin. Type of RCC not specified, grade II. Right nephrectomy and required 750 mg metformin daily at 2 month follow up.

IDDM – insulin dependent diabetes, AMS – altered mental status, DKA – diabetic ketoacidosis, GAD – glutamic acid decarboxylase, RCC – renal cell carcinoma.

period, the patient was found to have new, bilateral renal masses. The acute elevation of hemoglobin A1c to 11.3 mmol/mol is an atypical presentation of new onset diabetes in the absence of steroid use, severe pancreatitis, pancreatic cancer or pancreatectomy. Insulin therapy was required for 6 months with up to 43 units of long acting insulin per day at peak usage. Insulin requirements did appear to fluctuate rather dramatically, eventually requiring only glipizide. Following nephrectomy, the patient was able to remain on low dose oral hypoglycemic therapy as his hemoglobin A1c fell to 4.5 mmol/mol. This presentation and resolution of hyperglycemia following right-sided partial nephrectomy with embolization and cryoablation of the left sided mass is strongly suggestive of RCC as cause of the hyperglycemia.

A review of the literature revealed 7 previously reported cases of RCC with hyperglycemia in the English literature that were available for review (*4-10*). Years of publication ranged from 1981 to 2016. Available patient demographics and case details are included below (Table 1). One of the seven patients presented in diabetic ketoacidosis (DKA) (*5*). In all cases, there was improvement in glycemic control following nephrectomy. Both clear cell type and papillary type of RCC have been documented as causing these hyperglycemic states, however most case reports have not noted the histologic type of RCC. Furthermore, there are not enough cases reported to determine strength of correlation between RCC type and this hyperglycemic syndrome.

While the mechanism of hyperglycemia as a paraneoplastic syndrome in RCC has not yet been elucidated, a few possible mechanisms have been presented. One case noted increased levels of glucagon, which trended down after nephrectomy and presents the possibility of an endocrine mechanism of hyperglycemia (*6*). Another case detected elevation of anti-glutamic acid dehydroxylase (GAD) and anti-islet cell antibodies, which also normalized following nephrectomy and suggested a possible insulin deprivation mechanism due to pancreatic dysfunction on the level of the beta-islet cell (*5*). However, a review of the few other cases is not consistent with these previously noted findings. For instance, in the case of Harada *et al*, anti-GAD was tested for but was negative (*9*). For Calleawart and colleagues, glucagon, growth hormone, insulin like growth factor, cortisol and adrenocorticotropic hormone levels were all reported as normal (*4*). It may be that multiple mechanisms account for these few cases of significant hyperglycemia. More recent theories point to the effects of IL-6, which plays a role in other RCC paraneoplastic syndromes as well. In a study comparing cancer vs non-cancer patients, there was significantly decreased uptake of glucose in cancer patients with elevated IL-6 levels compared to those without elevated IL-6 (*11*). In other studies, injection of recombinant human IL-6 increased plasma glucose levels in a dose-dependent manner (*12*) and decreased insulin secretion from beta-islet cells (*13*).

Also, Blay and his colleagues studied the role of IL-6 levels in multiple paraneoplastic syndromes in patients with metastatic RCC and found that 90% of these patients had elevated IL-6 levels (*14*). Previous works by his group also showed expression of IL-6 from RCC cells in vitro as well. However, if 90% of patients with RCC have elevated levels of IL-6, the question of why so few cases of severe, new-onset hyperglycemia exist in RCC patients remains.

Hyperglycemia as a paraneoplastic syndrome in RCC is rare. The few reported incidences often show acute and severe hyperglycemia, which resolves with nephrectomy, as seen in the present case. These cases collectively indicate RCC as the most plausible cause of hyperglycemia. Further study is needed to determine the mechanism of hyperglycemia in RCC. While it is obvious that every episode of new onset hyperglycemia does not warrant a workup for RCC, new onset of severe hyperglycemia with common causes excluded and the presence of other signs of RCC might indicate a paraneoplastic etiology.

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(Received April 12, 2018; Revised May 12, 2018; Accepted May 19, 2018)

Case Report

DOI: 10.5582/irdr.2018.01050

Severe bacterial sepsis results in delayed diagnosis of tuberculous lymphadenitis in a rheumatoid arthritis patient treated with adalimumab

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Summary **Although tumor necrosis factor (TNF)-α inhibitors are effective in patients with rheumatoid arthritis (RA), an increased risk of infections often becomes a serious problem. It is well known that TNF-α inhibitors increase the risk of tuberculosis, but extrapulmonary tuberculosis often induced by them is difficult to diagnose using routine imaging examinations. We described a case of delayed diagnosis of a tuberculous lymphadenitis in a patient with RA treated with TNF-α inhibitor because of the complications of severe bacterial sepsis. In this case, rescreening with the interferon-γ release assay and excisional biopsy were useful in confirming the diagnosis of extrapulmonary tuberculosis. In the case we presented, she had other risk factors, that is, advanced age at the start of anti-TNF-α treatment or concomitant use of corticosteroid, might contribute to the development of complex infections. We should keep in mind that careful follow-up and appropriate examinations are necessary in caring for patients administering immunosuppressive treatments including anti- TNF-α drugs.**

Keywords: Tumor necrosis factor-α, septic shock, extrapulmonary tuberculosis, interferon-γ release assay

1. Introduction

Recently, the treatment for rheumatoid arthritis (RA) has changed drastically. Notably, biological drugs inhibiting tumor necrosis factor (TNF)-α have beneficial effects in patients with RA who are refractory to the conventional anti-rheumatoid therapy including disease-modifying antirheumatic drugs (DMARDs) (*1*). However, infections are the most common and important adverse effects of TNF-α inhibitors, because TNF-α is a key cytokine involved in cellular immunity (*2*). Especially, TNF-α plays an important role in host defense against *Mycobacterium tuberculosis* by mediating granuloma homeostasis and containment of latent disease (*3*). Therefore, prevention and early detection of tuberculosis (TB) are very important in patients with RA treated with TNF-α inhibitors (*4*). In this manuscript, we described a case of delayed diagnosis of a tuberculous lymphadenitis in a patient with RA treated with TNF-α inhibitor because of the complications of severe bacterial sepsis and septic shock.

2. Case Report

The patient was a 70-year-old woman who was diagnosed as having RA with polyarthritis at the age of 67 years. She underwent treatment with non-steroid anti-inflammatory drugs and sulfasalazine (up to 2,000 mg/day) for 1 year and then the treatment was changed to bucillamine (up to 300 mg/day) and prednisolone (5 mg/day). Methotrexate (MTX) was not prescribed because of deterioration of renal function. She had

Released online in J-STAGE as advance publication May 23, 2018.

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Table 1. Results of blood test

Items	Before GLM treatment	Before ADA treatment	At the onset of sepsis	When leaved ICU	When TB diagnosed	3weeks after TB treatment
WBC (× 10^9/L)	11.9	11.5	19.1	4.7	7.0	5.3
RBC (× 10^{12}/L)	4.07	3.68	4.66	4.11	4.15	4.01
Hb (g/L)	103	82	120	103	105	102
PLT (× 10^9/L)	335	531	255	168	256	206
AST (IU/L)	15	8	31	22	25	19
ALT (IU/L)	9	4	10	10	14	10
γ-GT (IU/L)	15	14	24	30	24	25
LD (IU/L)	213	201	379	268	282	242
CRE (μmol/L)	71.6	70.0	147.6	39.8	35.4	46.0
UN (mmol/L)	7.7	6.3	16.2	8.8	6.1	5.1
CK (IU/L)	20	16	636	29	11	8
CRP (mg/L)	49.7	100.6	182.8	23.7	44.7	16.6
PCT (μg/L)	-	-	28.96	0.59	< 0.1	-

WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; PLT, platelet; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GT, γ-glutamyl transpeptidase; LD, lactate dehydrogenase; UN, urea nitrogen; CK, creatine kinase; CRE, creatinine; CRP, C-reactive protein; PCT, procalcitonin.

also systemic sclerosis for 7 years and was visiting our hospital regularly. Because of the inefficiency of previous DMARDs treatment for longer than 3 years, anti-TNF-α treatment with golimumab (GLM; Simponi®) was introduced. She did not have history of TB or contact with a patient with TB, and the interferon-γ release assay (IGRA) was negative before starting GLM treatment. Six months after starting GLM, the patient still had had high RA activity (CRP level, 100.6 mg/L; pain visual analog scale (VAS) score, 80/100), and treatment was switched to adalimumab (ADA; Humira®). At first, the dose of ADA was 40 mg biweekly. After 1 month, the dose was increased to 80 mg biweekly, and then her RA activity gradually improved (CRP levels, 10.8 mg/L; VAS score, 35/100). Seven months after switching to ADA, the patient was taken to our hospital due to sudden high fever (up to 39.0ºC) with dyspnea, falling to hypotensive shock. On the assumption of severe septic shock, she was admitted in the intensive care unit (ICU). Other physical examinations revealed cervical and axillary lymphadenopathies and digital ulcers. A chest X-ray and whole-body computed tomography (CT) scan showed no identifiable source of infection. Laboratory examination results are presented in Table 1. No findings suggestive of the existence of fungal infection were identified. Owing to positive peripheral blood smear for gram-positive coccus, she was treated with a course of antibiotics (meropenem 1 g thrice daily, linezolid 600 mg once daily). As a result of a positive blood culture for methicillin-sensitive *Staphylococcus aureus*, the antibiotic was changed to cefazolin (2 g thrice daily) three days later. The antibacterial treatment described above improved her general medical condition; however, intermittent low fever, high levels of CRP (Table 1), and lymph node swelling (Figure 1A) persisted for a week. Provisional diagnosis at this point was to rule out underlying TB or lymphoma. Upon re-

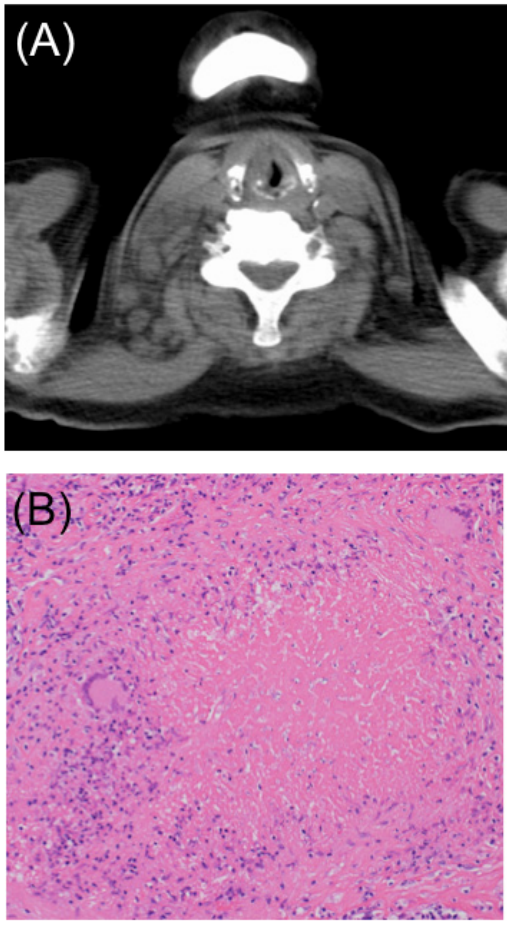

Figure 1. (A) Computed tomographic demonstrated multiple enlargements of right cervical lymph nodes. (B) Histological findings of lymph node specimen show granulomatous inflammation with caseous necrosis, multinucleate giant cells, and epithelioid cells. (hematoxylin-eosin stain; original magnification, 200×)

examination, the result of IGRA (quantiFERON®-TB gold) was positive. As the result of excisional biopsy of the cervical lymph node, histological findings

showed granulomatous inflammation with caseous necrosis, multinucleate giant cells, and epithelioid cells (Figure 1B). In addition, positive acid-fast bacilli were seen using Ziehl-Neelsen stain, and the patient was histopathologically diagnosed with tuberculous lymphadenitis. After starting antitubercular agents (isoniazid 300 mg thrice daily, rifampicin 450 mg once daily, pyrazinamide 1.2 g thrice daily, and ethambutol 750 mg thrice daily), she symptomatically improved and was later transferred to a hospital specializing in TB treatment.

3. Discussion

TNF-α has been implicated in the pathogenesis of many inflammatory diseases such as rheumatoid arthritis, psoriatic arthritis, and inflammatory bowel disease. Biological drugs that inhibit TNF-α have markedly changed the treatment and outcome of these diseases (*1*). ADA is one of the TNF-α inhibitors, which is the first fully human, high-affinity, anti-TNF-α monoclonal antibody. With or without MTX or other DMARDs, ADA provided enough evidence of effectiveness in the treatment of adults with refractory RA (*5*). Although anti-TNF-α treatment is drastically effective in refractory patients with RA, an increased risk of infections often becomes a serious problem (*2*). Due to the immunological role of TNF-α, the infection caused by anti-TNF-α treatment of the greatest concern is TB; the risk of TB in patients with RA receiving anti-TNF-α treatment has been documented to range from 0.2 to 4% (*6*). Previous reports estimated extrapulmonary TB to constitute more than 50% of cases of TB in patients treated with anti-TNF-α drugs (*4,7,8*). In the patients treated with ADA, Dixon et al. previously reported that disseminated disease was the most common (40%), although lymph node disease was relatively low (10%) (*7*). In a recent study, IGRA was found to be more specific and sensitive than the tuberculin skin test in patients with RA (*9*). In our case, however, despite the negative baseline screening with IGRA, the patient had converted IGRA 13 months after starting anti- TNF-α therapy. Although the clinical significance of IGRA conversions remains unclear, IGRA conversion was reportedly found in baseline IGRA-negative patients who developed TB late in the anti-TNF-α treatment (*10*). It is well known that TNF-α inhibitors increase the risk of TB, but extrapulmonary TB often induced by them is difficult to diagnose using a routine chest radiography or CT scanning. Hence, rescreening with IGRA is useful in suspected cases of TB, and excisional biopsy should be willingly performed to rule out extrapulmonary TB. In the case we presented, she had other risk factors, that is, advanced age at the start of anti-TNF-α treatment or concomitant use of corticosteroid, might contribute to the development of complex infections (*2*). We should keep in mind that careful follow-up and appropriate examinations are necessary in caring for patients administering immunosuppressive treatments including anti- TNF-α drugs.

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(Received May 2, 2018; Revised May 15, 2018; Accepted May 16, 2018)

Communication

DOI: 10.5582/irdr.2018.01018

Aurora B: A new promising therapeutic target in cancer

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Summary **A critical step for maintenance of genetic stability is chromosome segregation, which requires a high coordination of cellular processes. Loss of mitotic regulation is a possible cause of aneuploidy in human epithelial malignancy and it is thought to create an abnormal nuclear morphology in cancer cells. Serine/threonine protein kinase Aurora B gene plays a regulatory role from G2 to cytokinesis, encompassing key cell cycle events such as centrosome duplication, chromosome bi-orientation, and segregation. The overexpression of Aurora B has been observed in several tumour types, and has been linked with a poor prognosis for cancer patients. Therapeutic inhibition of Aurora kinase showed great promise as a probable anticancer regime because of its important role during cell division.**

Keywords: Aurora kinase, Aurora B, serine-threonine kinase, mitosis, cytokinesis, cancer, inhibitors

1. Aurora B and cancer

In the last decade, a large number of studies have linked the aberrant expression of Aurora kinases to cancer and this has lead to a great effort on the development of Aurora kinases inhibitors.

Aurora B is involved in chromosome segregation, spindle-checkpoint and cytokinesis, and alteration of each of these steps could induce aneuploidy, one of main features and driving force of cancer cells (*1-3*).

In vitro studies performed with several Aurora B inhibitors, dominant negative mutants or RNAi, show that Aurora B deficiency interferes with the cell cycle. Treated cells cannot divide after mitosis and become tetraploid, with two copies of centrosomes. Moreover, cells expressing catalytically inactive Aurora B do not arrest in mitosis in the presence of nocodazole or taxol. These observations concur with Aurora B's presumed roles: spindle checkpoint suppression allows cells to go through mitosis, despite a number of chromosomes being oriented in a syntelic manner (both kinetocores attached to the same pole), while the lack of phosphorylation of cleavage furrow components prevents cytokinesis. The effects of longer depletion of Aurora B seem to be cell line dependent. Some cells either enter additional cell cycles but, because of cell division failure, they become massively polyploid, whereas other cell lines undergo apoptosis or arrest in a pseudo G1 state. These differences are probably due to the p53-dependent post-mitotic checkpoint (*4-6*).

Aurora B is located on chromosome 17p13.1, a chromosomal region that has not been frequently associated with amplification in tumours, with the exception of glioblastoma (*7*). Aurora B gene is dramatically up-regulated in highly proliferating compared with non-proliferating cells. Although Aurora B overexpression has been shown in many tumours types, this is not the result of gene amplification, and it is still under debate whether the observed overexpression of Aurora B is a reflection of the high proliferative rate of neoplastic cells or whether it is causally related to tumorigenesis.

Aurora B is overexpressed in several human cancers, such as non small cell lung carcinoma (*8*), mesothelioma (*9*), glioblastoma (*7*) oral cancer (*10*), malignant endometrium, hepatocellular carcinoma (*11*), testicular germ cell tumours (*12-15*), ovarian (*16,17*), thyroid (*18,19*), colon (*20*) and prostate (*21,22*). Aurora B expression is positively correlated with poor prognosis and displays a tendency to group in higher grades of malignancy in different neoplastic lesions. Aurora B expression directly correlates with Gleason grade in prostate cancer (*21,22*), Duke's grade

Released online in J-STAGE as advance publication April 15, 2018.

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in colorectal cancer (*20*) and dedifferentiation in ovary and thyroid carcinoma (*16,18*). In thyroid tumours, an increase of Aurora B expression has been observed in papillary and anaplastic thyroid carcinomas. In the late stages of thyroid tumour progression a further increase of Aurora B expression was observed indicating that Aurora B overexpression might confer a growth advantage to neoplastic cells (*18,19*). In all lesions overexpressing Aurora B, phosphorylation of histone H3 was clearly detectable.

Several studies have suggested that commonly occurring gene polymorphisms of Aurora B are associated with cancer risk. An alternative splicing variant of Aurora B (Aurora B-Sv2) has been found frequently associated with advanced stages of hepatocellular carcinoma; and this variant appears to be more frequently associated with tumour recurrence and poor prognosis (*11*).

Aurora B kinase expression in epithelial ovarian cancer patients has been evaluated. Expression of Aurora B in poorly and moderately differentiated carcinomas of the ovary was significantly higher than in

Table 1. Clinical trials with Aurora kinase inihibitors

Drug	Tumor Type	Title of the Study	Phase	Sponsored by	ClinicalTrials.gov Identifier
VX-680 (MK0457)	Advanced Cancer	A Phase I, Open Label, Multi-centre Study to Assess the Safety, Tolerability, and Pharmacokinetics of AZD1152 in Japanese Patients With Acute Myeloid Leukaemia	Phase I	MERCK	NCT00104351
VX-680 (MK0457)	Leukemia	A Phase I/II Dose Escalation Study of MK0457 in Patients With Leukemia	Phase I	MERCK	NCT00111683
VX-680 (MK0457)	Carcinoma, Non-Small-Cell Lung	A Phase IIA Study Evaluating the Efficacy of MK0457 as a 5-Day Continuous Infusion in Patients With Advanced Non-Small Cell Lung Cancer (NSCLC)	Phase II	MERCK	NCT00290550
VX-680 (MK0457)	Leukemia	A Phase II Study of MK0457 in Patients With T315I Mutant Chronic Myelogenous Leukemia and Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia	Phase II	MERCK	NCT00405054
VX-680 (MK0457)	Chronic Myelogenous Leukemia, Leukemia, Lymphoblastic Acute Philadelphia Positive	A Phase I Dose Escalation of MK0457 in Combination With Dasatinib in Patients With Chronic Myelogenous Leukemia and Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia	Phase I	MERCK	NCT00500006
AZD1152	Acute Myeloid Leukemia	A Phase I, Open Label, Multi-centre Study to Assess the Safety, Tolerability, and Pharmacokinetics of AZD1152 in Japanese Patients With Acute Myeloid Leukaemia	Phase I	AstraZeneca	NCT00530699
AZD1152	Myeloid Leukemia	A Phase I/II, Open Label, Multi-Centre Study to Assess the Safety, Tolerability, Pharmacokinetics and Efficacy of AZD1152 in Patients With Acute Myeloid Leukaemia	Phase I Phase II	AstraZeneca	NCT00497991
AZD1152 + LDAC	Acute Myeloid Leukemia	A Randomised, Open-label, Multi-centre, 2- stage, Parallel Group Study to Assess the Efficacy, Safety and Tolerability of AZD1152 Alone and in Combination With Low Dose Cytosine Arabinoside (LDAC) in Comparison With LDAC Alone in Patients Aged 60 With Newly Diagnosed Acute Myeloid Leukaemia (AML)	Phase II	AstraZeneca	NCT00952588
AZD1152 + LDAC	Acute Myeloid Leukemia	A Phase I, Open-Label, Multi-Centre, Multiple Ascending Dose Study to Assess the Safety and Tolerability of AZD1152 in Combination With Low Dose Cytosine Arabinoside (LDAC) in Patients With Acute Myeloid Leukaemia (AML)	Phase I	AstraZeneca	NCT00926731
AZD1152	Solid Tumours	A Phase I, Open-Label, Multi-Centre Study to Assess the Safety, Tolerability and Pharmacokinetics of AZD1152 Given as a Continuous 48-Hour Intravenous Infusion in Patients With Advanced Solid Malignancies	Phase I	AstraZeneca	NCT00338182
AZD1152	Solid Tumours	A Phase I, Open-Label, Multi-Centre Study to Assess the Safety, Tolerability and Pharmacokinetics of AZD1152 Given as a Continuous 7- Day Intravenous Infusion in Patients With Advanced Solid Malignancies	Phase I	AstraZeneca	NCT00497679

well-differentiated carcinomas and overall, the Aurora B overexpression group demonstrated a significantly shorter progression-free survival and survival than a low expression group (*16*). In human colorectal cancer samples the correlation of Aurora B expression with overall survival was also evaluated, showing that patients with a high expression level of Aurora B lived significantly shorter lives compared with patients with low expression levels . Furthermore single-nucleotide polymorphism analysis showed that patients harboring G-allele in 885A>G showed a significantly decreased overall survival (*1*). These studies suggest that Aurora B expression could be used as a predictor of aggressive lesions and as a prognostic marker.

2. Aurora B as therapeutic target

The inhibition of Aurora B kinase has an anti-proliferative effect and causes regression in several animal models of human cancers, including breast, colon, lung, leukemia, prostate and thyroid (*15-22*). These observations strongly suggest a potential role for Aurora B inhibition in patients. To target the enzymatic activity of Aurora kinases, small molecules able to occupy the catalytic binding site have been identified, however due to the high homology of Aurora kinases catalytic subunit most of the inhibitors developed so far lack selectivity and inhibit both Aurora Kinases. Despite this lack of selectivity of Aurora Kinases inhibitors the number of Aurora inhibitors is rapidly increasing: approximately 15 Aurora inhibitors are under Phase I/II evaluation and others are in preclinical testing (Table 1) (*3,23*).

Inhibition of Aurora B by specific inhibitors interferes with normal chromosome alignment during mitosis and overrides the mitotic spindle checkpoint inducing endoreduplication. Aurora B seems to be more suitable as an anticancer drug target, since inhibition of Aurora B rapidly results in catastrophic mitosis, leading to cell death (*23-28*).

In order to block Aurora B functions, several anti-cancer drugs targeting its catalytic binding site have been developed. However, given the high homology of the catalytic domain of Aurora kinases, small molecules do not differentiate between them, and could be considered dual inhibitors (Aurora A and B inhibithors), however the phenotype observed in cells treated with these dual inhibitors closely resemble those exerted by specific inhibition of Aurora B. The first three small-molecule dual inhibitors of Aurora(s) described include Hesperadin, VX-680, and ZM447439. These three drugs have similar potency versus Aurora A, and Aurora B. Each induces a similar phenotype in cell-based assays, characterized by inhibition of phosphorylation of histone H3 on Ser^{10} and Ser^{28}, inhibition of cytokinesis, and development of polyploidy (*28-34*).

In addition, selective inhibitors of Aurora B have been developed: AZD1152 (highly potent and selective inhibitor of Aurora B), and GSK1070916 (a potent and selective inhibitor of Aurora B and Aurora C kinases) (*3,19,23*).

The role of Aurora B in human tumourigenesis remains to be fully clarified, however, overexpression of Aurora B occurs in many tumours and accumulating evidence indicates that its expression negatively correlates with patient's survival and prognosis.

Aurora B inhibitors have been obtained and are currently under clinical trials. However, antimitotic drugs frequently show the appearance of drug resistance, adverse side effects (neurotoxicity) and their clinical effects are not predictable. Aurora inhibitors lack neurotoxicity and could contribute to the treatment of human neoplasia (*31-34*).

Furthermore, preclinical data are predicting that the association of these inhibitors with conventional chemotherapy and radiotherapy display additive or even synergistic anticancer effect thus opening new avenues towards the cure of cancer.

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(Received February 20, 2018; Revised March 13, 2018; Accepted March 24, 2018)

Communication

DOI: 10.5582/irdr.2018.01056

China has officially released its first national list of rare diseases

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Summary **Over the past few years, the Chinese Government has paid greater attention to rare diseases and it has incorporated rare diseases in national health strategy and planning. On May 22, 2018, the Chinese Government officially released its first list of rare diseases, which included 121 rare diseases. The list was published to facilitate greater societal awareness of rare diseases, to improve the ability of front-line medical staff to treat rare diseases, to introduce incentives for research and development of orphan drugs, and to increase the availability of medicines for rare diseases. This effort will enhance the management of rare diseases in China, raise the level of diagnosis and treatment for rare diseases, and safeguard the health-related rights and interests of patients with rare diseases. The classification of rare diseases is based on a common international standards, which will promote international cooperation in drug research and policymaking with regard to rare diseases.**

Keywords: Rare diseases, disease classification, disease management, orphan drugs

On May 22, 2018, China's *First National List of Rare Diseases* (hereinafter referred to as the Chinese Rare Diseases List, CRDL) was issued jointly by five national bodies, including the National Health Commission, Ministry of Science and Technology, Ministry of Industry and Information Technology, State Drug Administration, and State Administration of Traditional Chinese Medicine (*1*). The CRDL gives priority to rare diseases with a relatively high prevalence, that pose a heavy burden, and that are highly treatable. The CRDL includes a total of 121 rare diseases (Table 1), such as albinism, amyotrophic lateral sclerosis, Gaucher's disease, Kallmann syndrome, Marfan syndrome, Fabry disease, and hemophilia.

The CRDL represents a clear "list of rare diseases" as mentioned in the *Opinions on Further Reform of the Review and Approval System to Encourage Innovation in Drugs and Medical Devices* issued by the former State Administration of Food and Drug Administration of China in October 2017 (*2*). Publication of the CRDL will help China enhance its management of rare diseases, improve the diagnosis and treatment of rare diseases, and safeguard the health-related rights of patients with rare diseases. The CRDL will serve as a reference for relevant government agencies and ministries in the future.

Thus far, rare diseases have not been officially defined in China. The definition in popular use was based on a consensus of experts reached by the Genetics Branch of the Chinese Medical Association in May 2010. According to this definition, a rare disease is a disease with a prevalence of less than 1/500,000 or a neonatal morbidity of less than 1/10,000 (*3*). Since epidemiological data on rare diseases are lacking in China, the current list of rare diseases is based on actual conditions, and the list is mainly derived from the professional opinions of the *Expert Committee on the Diagnosis, Treatment, and Care for Rare Diseases* established by the Medical Administration Bureau of the former National Health and Family Planning Commission.

As early as 2016, the Shanghai Health and Family Planning Commission published the first local list of rare diseases in China entitled *The List of Major Rare Diseases in Shanghai (2016 Edition)*. The Shanghai list which includes 56 rare diseases, 50 of which are also included in the CRDL (*4*). On September 23

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Table 1. The first national list of rare diseases in China*

No.	Diseases	No.	Diseases
1	21-Hydroxyulase Deficiency	60	Langerhans Cell Histiocytosis
2	Albinism	61	Laron Syndrome
3	Alport Syndrome	62	Leber Hereditary Optic Neuropathy
4	Amyotrophic Lateral Sclerosis	63	Long Chain 3-hydroxyacyl-CoA Dehydrogenase Deficiency
5	Angelman Syndrome	64	Lymphangioleiomyomatosis (LAM)
6	Arginase Deficiency	65	Lysine Urinary Protein Intolerance
7	Asphyxiating Thoracic Dystrophy (Jeune Syndrome)	66	Lysosomal Acid Lipase Deficiency
8	Atypical Hemolytic Uremic Syndrome	67	Maple Syrup Urine Disease
9	Autoimmune Encephalitis	68	Marfan Syndrome
10	Autoimmune Hypophysitis	69	McCune-Albright Syndrome
11	Autoimmune Insulin Receptopathy (Type B insulin resistance)	70	Medium Chain Acyl-CoA Dehydrogenase Deficiency
12	Beta-ketothiolase Deficiency	71	Methylmalonic Academia
13	Biotinidase Deficiency	72	Mitochondrial Encephalomyopathy
14	Cardic Ion Channelopathies	73	Mucopolysaccharidosis
15	Carnitine Deficiency	74	Multi-Focal Motor Neurothy
16	Castleman Disease	75	Multiple Acyl-CoA Dehydrogenase Deficiency
17	Charcot-Marie-Tooth Disease	76	Multiple Sclerosis
18	Citrullinemia	77	Multiple System Atrophy
19	Congenital Adrenal Hypoplasia	78	Myotonic Dystrophy
20	Congenital Hyperinsulinemic Hypoglycemia	79	NAGS Deficiency
21	Congenital Myasthenic Syndrome	80	Neonatal Diabetes Mellitus
22	Congenital Myotonia Syndrome (Non-Dystrophic Myotonia, NDM)	81	Neuromyelitis Optica
23	Congenital Scoliosis	82	Niemann-Pick Disease
24	Coronary Artery Ectasia	83	Non-Syndromic Deafness
25	Diamond-Blackfan Anemia	84	Noonan Syndrome
26	Erdheim -Chester Disease	85	Ornithine Transcarbamylase Deficiency
27	Fabry Disease	86	Osteogenesis Imperfecta (Brittle Bone Disease)
28	Familial Mediterranean Fever	87	Parkinson Disease (Young-onset, Early-onset)
29	Fanconi Anemia	88	Paroxysmal Nocturnal Hemoglobinuia
30	Galactosemia	89	Peutz-Jeghers Syndrome
31	Gaucher's Disease	90	Phenylketonuria
32	General Myathenic Gravis	91	POEMS Syndrome
33	Gitelman Syndrome	92	Porphyria
34	Glutaric Acidemia Type I	93	Prader-Willi Syndrome
35	Glycogen Storage Disease (Type I, II)	94	Primary Combined Immune Deficiency
36	Hemophilia	95	Primary Hereditary Dystonia
37	Hepatolenticular Degeneration (Wilson Disease)	96	Primary Light Chain Amyloidosis
38	Hereditary Angioedema (HAE)	97	Progressive Familial Intrahepatic Cholestasis
39	Hereditary Epidermolysis Bullosa	98	Progressive Muscular Dystrophies
40	Hereditary Fructose Intolerance	99	Propionic Acidemia
41	Hereditary Hypomagnesemia	100	Pulmonary Alveolar Proteinosis
42	Hereditary Multi-infarct Dementia (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalqpathy, CADASIL)	101	Pulmonary Cystic Fibrosis
		102	Retinitis Pigmentosa
		103	Retinoblastoma
43	Hereditary Spastic Paraplegia	104	Severe Congenital Neutropenia
44	Holocarboxylase Synthetase Deficiency	105	Severe Myoclonic Epilepsy In Infaricy (Dravet Syndrome)
45	Homocysteinemia	106	Sickle Cell Disease
46	Homozygous Hypercholesterolemia	107	Silver-Russell Syndrome
47	Huntington Disease	108	Sitosterolemia
48	Hyperornithinaemia-Hyperammonaemia-Hhomocit rullinuria Syndrome	109	Spinal and Bulbar Muscular Atrophy (Kennedy Disease)
		110	Spinal Muscular Atrophy
49	Hyperphenylalaninemia	111	Spinocerebellar Ataxia
50	Hypophosphatasia	112	Systemic Sclerosis
51	Hypophosphatemia Rickets	113	Tetrahydrobiopterin Deficiency
52	Idiopathic Cardiomyopathy	114	Tuberous Sclerosis Complex
53	Idiopathic Hypogonadotropic Hypogonadism	115	Tyrosinemia
54	Idiopathic Pulmonary Arterial Hypertension	116	Very Long Chain Acyl-CoA Dehydrogenase Deficiency
55	Idiopathic Pulmonary Fibrosis	117	Williams Syndrome
56	IgG4 related Disease	118	Wiskott-Aldrich Syndrome
57	Inborn Errors of Bile Acid Synthesis	119	X-linked Agammaglobulinemia
58	Isovaleric Acidemia	120	X-linked Ldrenoleuko Dystrophy
59	Kallmann Syndrome	121	X-linked Lymphoproliferative Disease

*From the *Notice on the First National List of Rare Diseases in China* jointly issued by five bodies, including the National Health Commission. (*1*).

of the same year, a social organization, the Chinese Organization for Rare Disorders (CORD), published the *Reference List of Rare Diseases in China* (*5*). The list includes 147 rare diseases, 88 of which are also included in the CRDL.

Currently, there are no specific policies on rare diseases or orphan drugs nationwide, but the area of rare diseases has received increasing attention in recent years. The central government has included rare diseases in major health planning and strategy, including the *five-year plan on public healthcare (2016-2020)* (*6*) and the *"Healthy China 2030" Planning Outline* (*7*). Local government has actively promoted care for rare diseases. In Qingdao, a city in Shandong Province, medical insurance has covered Behcet syndrome, multiple sclerosis, and myasthenia gravis since 2005 (*8*). In Shanghai, the Children's Hospitalization Fund has covered Pompeii disease, Gaucher's disease, mucopolysaccharidosis, and Fabry disease since 2011 (*9*). In Zhejiang Province, medical insurance has covered Gaucher's disease, amyotrophic lateral sclerosis, and phenylketonuria since 2016 (*10*).

Issuance of the CRDL has resolved the issue of "no official definition, no specific policy support, and no coverage by medical insurance" of rare diseases in China. The list facilitates greater societal awareness of rare diseases, it improves the ability of medical personnel to diagnose and treat rare diseases, it furnishes incentives for research and development of orphan drugs, and it increases the affordability of orphan drugs. The classification of rare diseases is based on an international consensus, which is sure to promote international cooperation in clinical trials and healthcare policymaking in the area of rare diseases.

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(Received May 27, 2018; Accepted May 29, 2018)

IRDR
Intractable & Rare Diseases Research

Guide for Authors

1. Scope of Articles

Intractable & Rare Diseases Research is an international peer-reviewed journal. Intractable & Rare Diseases Research devotes to publishing the latest and most significant research in intractable and rare diseases. Articles cover all aspects of intractable and rare diseases research such as molecular biology, genetics, clinical diagnosis, prevention and treatment, epidemiology, health economics, health management, medical care system, and social science in order to encourage cooperation and exchange among scientists and clinical researchers.

2. Submission Types

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