ISSN 2186-3644 Online ISSN 2186-361X

IRDR

Intractable & Rare Diseases Research

Volume 10, Number 1
February, 2021

IRCA
BSSA

IACMHR

www.irdrjournal.com

IRDR
Intractable & Rare Diseases Research

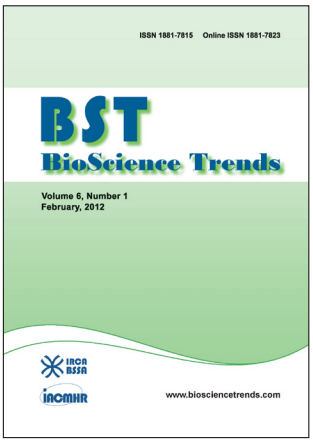
ISSN: 2186-3644
Online ISSN: 2186-361X
CODEN: IRDRA3
Issues/Year: 4
Language: English
Publisher: IACMHR Co., Ltd.

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.

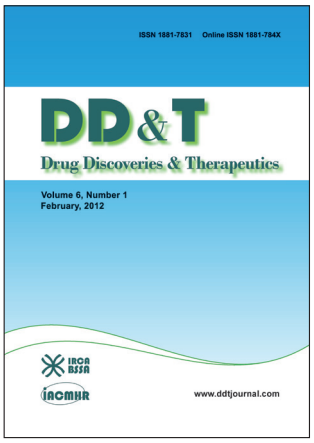
Intractable & Rare Diseases Research devotes to publishing the latest and most significant research in intractable and rare diseases. Articles cover all aspects of intractable and rare diseases research such as molecular biology, genetics, clinical diagnosis, prevention and treatment, epidemiology, health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

Intractable & Rare Diseases Research publishes Original Articles, Brief Reports, Reviews, Policy Forum articles, Case Reports, Communications, Editorials, News, and Letters on all aspects of the field of intractable and rare diseases research. All contributions should seek to promote international collaboration.

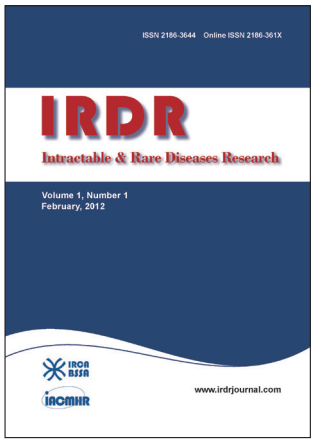
IRCA-BSSA Group Journals



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

Editorial and Head Office

Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan

E-mail: office@irdrjournal.com
URL: www.irdrjournal.com

Editorial Board

CONTENTS

Volume 10, Number 1, 2021

Review

Original Article

Brief Report

Case Report

Letter

Review

DOI: 10.5582/irdr.2020.03077

Perspectives on urological care in spina bifida patients

Mohamad Moussa[1], Athanasios G. Papatsoris[2], Mohamad Abou Chakra[3,*], Youssef Fares[4], Baraa Dabboucy[5], Athanasios Dellis[6]

[1] Urology Department, Zahraa Hospital, University Medical Center, Lebanese University, Beirut, Lebanon;
[2] 2nd Department of Urology, School of Medicine, Sismanoglio Hospital, National and Kapodistrian University of Athens, Athens, Greece;
[3] Department of Urology, Faculty of Medicine, Lebanese University, Beirut, Lebanon;
[4] Department of Neurosurgery, Neuroscience Research Center, Faculty of Medical Sciences, Lebanese University, Beirut, Lebanon;
[5] Department of Neurosurgery, Faculty of Medicine, Lebanese University, Beirut, Lebanon;
[6] Department of Urology/General Surgery, Areteion Hospital, Athens, Greece.

SUMMARY Spina bifida (SB) is a neurogenetic disorder with a complex etiology that involves genetic and environmental factors. SB can occur in two major forms of open SB or SB aperta and closed SB or SB occulta. Myelomeningocele (MMC), the most common neural tube defects (NTDs), occurs in approximately 1 in 1,000 births. Considering non-genetic factors, diminished folate status is the best-known factor influencing NTD risk. The methylenetetrahydrofolate reductase (MTHFR) gene has been implicated as a risk factor for NTDs. The primary disorder in the pathogenesis of MMC is failed neural tube closure in the embryonic spinal region. The clinical manifestation of SB depends on clinical type and severity. SB can be detected in the second trimester using ultrasound which will reveal specific cranial signs. The management of MMC traditionally involves surgery within 48 h of birth. Prenatal repair of MMC is recommended for fetuses who meet maternal and fetal Management of Myelomeningocele Study (MOMS) specified criteria. Urological manifestations of SB include urinary incontinence, urolithiasis, sexual dysfunction, renal dysfunction, and urinary tract infection. Renal failure is among the most severe complications of SB. The most important role of the urologist is the management of neurogenic bladder. Medical management with clean intermittent catheterization and anticholinergic treatment is generally considered the gold standard of therapy. However, when this therapy fails surgical reconstruction become the only remaining option. This review will summarize the pathogenesis, risk factors, genetic contribution, diagnostic test, and management of SB. Lastly, the urologic outcomes and therapies are reviewed.

Keywords spina bifida, neuropathic bladder, myelomeningocele, urology

1. Introduction

Spina bifida (SB) is the most common birth defect affecting the central nervous system. The most common form of SB is myelomeningocele (MMC). MMC usually affects the brain with characteristic phenotypic features that involve cognition, behavior, and adaptation (*1*). SB is a congenital malformation in which the spinal column is split (bifid) as a result of failed closure of the embryonic neural tube, during the fourth week post-fertilization. EUROCAT, the European network of population-based registries for epidemiological surveillance of congenital anomalies estimated the prevalence (including chromosomally-related disorders) of 'SB' and 'neural tube defects' (NTDs) at 0.51 and 0.94 respectively per 1,000 births, stillbirths and pregnancy terminations (*2*). A study done in Malaysia showed that the prevalence of NTDs was 0.42 per 1,000 live births (*3*). In a systemic review, the overall birth prevalence of NTDs in India was 4.1 per 1,000 (*4*). Data for 2000 to 2014 in five counties in northern China were obtained through a population-based birth defects surveillance system. The prevalence of total NTDs was extremely high from 2000 to 2004, but it began to decrease continuously thereafter, from a peak of 120.0/10,000 in 2004 to a low of 31.5/10,000 in 2014 (*5*). In other areas, the reported NTD prevalence ranges and medians for each region were: African (5.2-75.4; 11.7 per 10,000 births), Eastern Mediterranean (2.1-124.1; 21.9 per 10,000 births) (*6*).

The causes of this disorder are heterogeneous and include chromosome abnormalities, single-gene disorders, and teratogenic exposures. However, the cause is not known in most cases. Up to 70% of SB cases can be prevented by maternal, periconceptional folic acid

supplementation (*7*). Most neurological dysfunctions related to MMC are already well established in adult ages, but new and more debilitating clinical problems can appear. Autonomic dysfunction, particularly from the bladder and bowel, remains a challenge also for persons with SB in adulthood (*8*). MMC management includes life-long comprehensive neurologic, urologic, musculoskeletal, skin, and rehabilitation management (*9*).

Urological manifestation in patients with MMC is common, resulting in serious negative psychological and medical effects. This mandates an early follow-up, and a comprehensive management plan to prevent any irreversible renal damage and stabilize bladder function (*10*). Despite consensus regarding early urological involvement in the care of patients with SB, controversy remains regarding optimal management. Major reconstructive urological surgeries still have a major role in the management of these cases to protect the upper urinary tract and to achieve continence (*11*). The urologist plays an important role in the multidisciplinary team of physicians who provide care for patients with SB. The essential role of the urologist is to prevent deterioration of kidney function and ensuring adequate bladder voiding. To achieve those goals medical and surgical therapies are used such as clean intermittent catheterization (CIC), antimuscarinic and urinary tract reconstruction (*12*).

We performed a narrative review to discuss briefly the etiology, pathophysiology, diagnosis, and treatment of SB. The urologist had a crucial role in the management of patients with SB. Understanding the pathogenesis of SB enables optimization of the management of urologic problems created by this malformation. We reviewed the current literature regarding the urological outcomes and management of patients with SB.

2. Overview of SB

2.1. Pathogenesis

On the basis of the presence or absence of overlying skin covering, spinal dysraphism is divided into open and closed types. In Open spinal dysraphism (OSD) overlying skin covering is absent and the neural elements are exposed to the external environment whereas, in the closed type, the neural elements have a skin covering. OSD results from faulty primary neurulation due to defective closure of the neural tube. About 98.8% of all OSDs are made up of MMC. Other entities of OSD include myelocele and hemimyelocele. Closed spinal dysraphism include meningocele, dermal sinus, and complex dysraphic states (*13*). There are two fundamental theories regarding the embryogenesis of MMC both encompassing a disorder of primary neurulation. In the so-called non-closure theory initially suggested by von Recklinghausen, it is proposed that neural tube defects represent a primary failure of neural tube closure. In the overdistension theory, introduced in 1769 by Morgagni and popularized by Gardner, it is proposed that NTDs arise through overdistension and rupture of a previously closed neural tube. The non-closure theory is more widely accepted and certainly accounts for the majority of human NTDs (*14*).

The neural damage in MMC may be primarily the result of defective spinal cord development, a secondary event resulting from damage to the exposed spinal cord by the intrauterine milieu. The two-hit hypothesis states that primary congenital abnormalities in anatomic development allow a relatively normal spinal cord to become secondarily damaged by amniotic fluid exposure, direct trauma, hydrodynamic pressure, or a combination of these factors (*15*). If the progression of spinal neurulation along the body axis is severely delayed or halted, then open SB results. In normal embryos, the vertebral arches develop from the sclerotomal component of the axial mesoderm, which migrates dorsally to surround the closed neural tube before undergoing cartilaginous and bony differentiation. When the neural folds remain open, the sclerotome is unable to cover the neuroepithelium and a bifid vertebral column is a secondary result (*16*). In summary, the neurologic defects in MMC result from primary incomplete neurulation and secondary chronic prenatal damage to the exposed neural elements through mechanical and chemical trauma (*17*).

Meningocele is often described as a less severe variant of MMC in which the spinal cord is not found in the sac and is described by embryologists to be absent of neural matter in its herniated sac. MMC is usually associated with a type II Chiari hindbrain malformation, ventriculomegaly, and hydrocephalus (*18*). Some structural anomalies are virtually unique to individuals with MMC, including a complex pattern of cerebellar dysplasia known as the Chiari II malformation. Other structural anomalies are not necessarily unique to MMC, including altered development of the corpus callosum and posterior fossa (*19*). The Chiari malformation is associated with hindbrain herniation, which may be caused by low spinal pressure relative to cranial pressure. The relationship between hydrocephalus and SB has been the subject of prolonged debate. A hypothesis proposed in this essay supports the view that SB is a manifestation of progressive hydrocephalus in the fetus. It is proposed that that mesodermal growth insufficiency influences both neural tube closure and central nervous system pressure, leading to dysraphism (*20*). An open neural tube defect allows fluid to escape from the cranial vesicles, altering the intracranial environment and leads to all of the brain changes seen in the Chiari II malformation. Decompression of the intracranial vesicles causes overcrowding, a decrease in the size of the third ventricle, and changes in the fetal skull (*21*). Hydrocephalus usually develops secondary to impaction of the posterior fossa contents on the foramen magnum, leading to occlusion of the outlets of the fourth ventricle

Table 1. Pathogenesis and characteristics of each type of spina bifida

Type of Spina Bifida	Characteristics	Pathogenesis
Myelomeningocele	Spinal cord is not found in the sac	Non closure theory: Primary failure of neural tube closure.
Meningocele	Neural matter herniating at the site of the lesion	Overdistension theory: Overdistension and rupture of a previously closed neural tube.
Spina bifida occulta	Site of the lesion is covered with skin	Defective secondary neurulation.

Table 2. Risk factors for spina bifida

Maternal factors
Not taking folic acid supplements
Spina bifida patients within third-degree relatives
Antiepileptic drugs
Pregestational diabetes
Gestational diabetes
Obesity
Vitamin B_{12} deficiency
Other factors
Low birth weight in the newborn
Pesticides
Paternal exposure to Agent Orange

with cerebrospinal fluid outflow blocked, or impaired, at the foramina of Luschka and Magendie and resulting in progressive ventriculomegaly. Although there are several theories, it has been demonstrated that 80-90% of children born with MMC are affected with Chiari II malformation, aqueductal stenosis, or fourth ventricular outflow obstruction causing non-communicating hydrocephalus (*22*).

A high number of patients with MMC also suffer from spinal cord tethering (SCT), which progressively worsens neurological function and frequently requires surgical correction (*23*). Approximately 10 to 30% of children will develop SCT following repair of a MMC. Because essentially all children with repaired MMC will have a SCT, as demonstrated on Magnetic resonance imaging (MRI), the diagnosis of tethered cord syndrome (TCS) is made based on clinical criteria (*24*).

There is also a less well-defined group of closed spinal NTDs in which the vertebral arches are malformed but covered by skin. These conditions, including SB occulta and spinal dysraphisms, vary widely in clinical presentation. The more severe subtypes are associated with various abnormalities of the spinal cord, lipoma, and/or anorectal abnormalities (*25*). SB occulta has an overall prevalence of 12.4% in a large, diverse population. SB occulta is more common in men and decreases in prevalence with increasing age (*26*). Closed spinal dysraphism, SB occulta, refers to skin-covered lesions. The cutaneous stigmata that may indicate an underlying dysraphism are particularly hairy patches, subcutaneous lipomas, capillary hemangiomas, dorsal dermal sinuses, and sacral cutaneous pits (*27*). The pathogenesis and the characteristics of each type of SB are summarized in Table 1.

A routine screening for other malformations especially facial clefts, musculoskeletal, renal and cardiac anomalies may need to be considered in infants with NTDs, and genetic counseling seems warranted in most of these complicated cases (*28*).

2.2. Risk factors

Many factors determining SB risks were cited in a comprehension overview, which included third pregnancy, miscarriage, high emotional stress during pregnancy, TORCH (toxoplasmosis, other agents, rubella, cytomegalovirus, and herpes) infection when pregnant, poor housing and maternal age (*29*). Risk factors for SB are summarized in Table 2.

In a study, four variables were significantly associated with the increased risk of having newborns afflicted with SB: not taking folic acid supplements, presence of SB patients within third-degree relatives, taking anti-epileptic drugs without folic acid, and low birth weight in the newborns ≤ 2,500 grams (*30*). Other factors are known or highly suspected to increase the risk for NTDs, including female infant sex and family history of NTDs, as well as maternal Hispanic ethnicity, obesity, pregestational diabetes, gestational diabetes, and hot tub or sauna use (*31*). Folic acid not only prevents the occurrence of a significant proportion of NTDs, but might also decrease the severity of NTDs as long as supplementation is started before conception (*32*).

Currently, strong evidence exists to suggest a causal association for maternal obesity before pregnancy, and paternal exposure to Agent Orange in patients with SB (*33*). Other risk factors for NTD are exposure to certain medications (valproic acid), and vitamin B_{12} deficiency. It was recommended that all women of childbearing age capable of becoming pregnant consume 400 micrograms of folic acid daily to prevent NTD's (*34*). NTDs are a complex disease impacted by genetic susceptibility, epigenetic influences, and environmental insults. Tools are now available to identify genetic contributions in humans using unbiased methods to evaluate the genome and epigenome (*35*).

2.3. Genetic contribution to SB

Disturbance of any of the sequential events of embryonic neurulation produces NTDs, with the phenotype (anencephaly, SB) varying depending on the region of the neural tube that remains open. While mutation of > 200 genes is known to cause NTDs in mice, the pattern of occurrence in humans suggests a

multifactorial polygenic or oligogenic etiology (*36*). The genes contributing to the etiology of NTDs are unknown. Mutations in planar cell polarity (PCP) genes in mice cause a variety of defects including the NTD, craniorachischisis, and sometimes SB. Recent studies have sought rare predicted-to-be-deleterious alterations (putative mutations) in the coding sequence of PCP genes in human cases with various anomalies of the neural tube. PCP rare putative mutations had a weaker role in MMC, being found in approximately 6% of cases and cumulated across CELSR1, FUZ, FZD6, PRICKLE1, VANGL1, and VANGL2 (*37*). Genetic variation might interact in a digenic fashion to generate visible NTD phenotypes and emphasize the importance of these genetic interactions in the development of NTDs in humans (*38*). The Wnt/PCP pathway remains a genetic hotspot. Addressing these issues is essential for understanding the genetic etiology of human NTDs. Data indicate rare damaging variants of the CELSR genes, identified in ~14% of NTD cases, and are expected to be driver genes in the Wnt/PCP pathway (*39*).

Several studies have found a positive association between NTDs and the common mutation 677C > T of 5,10-methylenetetrahydrofolate reductase (MTHFR), and others that have not indicated such an association (*40*). The enzyme MTHFR plays a key role in the folate metabolism pathway and regulates the intracellular folate pool for synthesis and methylation of DNA. The MTHFR gene is located at chromosome 1p36.3. It is assumed that MTHFR genetic polymorphisms play an important role in the development of NTDs; however, only 13% of NTDs were attributed to the MTHFR C677T mutation suggesting that the MTHFR C677T polymorphism alone cannot be responsible for NTDs (*41*). The combination of MTHFR and cystathione-b-synthase (CBS) mutations was reported to have a fivefold increase in the risk for SB compared with each variant alone, indicating the presence of gene-gene interactions (*42*). Another single nucleotide polymorphism (SNP) in the *MTHFR* gene, A1298C, has also been described and studied for its relationship to NTDs. Available data suggest that the A1298C variant alone is probably not a major risk factor for MMC. Data also suggest that compound heterozygosity for the C677T and A1298C alleles might be associated with an increased risk for MMC (*43*). Significant association of SNP (rs3737965) in MTHFR was found. *MTHFR* rs3737965 is located in the promoter sequence and therefore variants may affect transcriptional activity. This SNP was found to be associated with SB risk (*44*).

The identification of genetic risk factors for human NTDs is complicated by the multiplicity of genes participating in neurulation, and the importance of gene-environment interactions. Gene-environment interactions appear likely to contribute to NTD predisposition, with examples including interactions of MTHFR with multivitamin use, methionine synthase reductase (MTRR) with vitamin B_{12} and platelet derived growth factor receptor alpha (PDGFRA) with inositol and zinc (*45*).

2.4. Diagnostic test for SB

Prenatal screening for neurological abnormalities is based on an ultrasound performed routinely or oriented by maternal Alpha Feto Protein (AFP) screening. It should be performed around 12, 22, and 32 weeks. Maternal serum screening can detect up to 80% of cases of SB (*46*). Standard ultrasound improved NTD detection over AFP screening alone, by improving AFP test sensitivity and identifying NTDs in low-risk pregnancies (*47*). Compared with maternal serum AFP performed alone for screening, routine second-trimester ultrasonography was more likely to discover a NTD (*48*). Ultrasound-detectable signs of open SB include "banana sign" of the cerebellum and "lemon sign" of the frontal skull. A chromosomal abnormality was found in 10.9% of isolated SB, which is comparable to the rates reported in similar studies. This suggests that there is a high risk of chromosomal anomalies in these pregnancies compared with normal-appearing fetuses (*49*).

Ultrasound examination is the gold standard for the diagnosis of SB aperta. It represents the main imaging tool used to ascertain this diagnosis early in gestation. Three-dimensional ultrasound is necessary to detect the level and size of the defect. MRI represents a more sensitive tool, giving specific information on the defect and associated anomalies, playing an important role in ruling out the differential diagnosis (*50*). In tertiary fetal medicine centers, two-dimensional and three-dimensional ultrasound allows an accurate determination of the location, type, extent, and upper level of the spinal defect as well as the presence of associated anomalies. Fetal MRI should be restricted to candidates for intrauterine surgery as part of the preoperative protocol (*51*). Fetal MRI has advanced rapidly in the last 25 years, developing from an experimental technique to become a fundamental tool in normal clinical practice in many centers around the world. MRI's ability to detect complex anomalies that involve different organs has been widely reported (*52*).

During the prenatal evaluation, detailed ultrasonographic assessment of the entire spine with the identification of the position and morphology of the conus medullaris and absence of cranial signs of spinal dysraphism are the most valuable sonographic clues for diagnosis of closed SB (*53*). Additional imaging in the postnatal period can be useful in evaluating the newborn with vertebral anomalies noted on prenatal imaging. Plain radiographs (anteroposterior and lateral of the entire spine including the ribs), should be obtained early, optimally in the first 2 months, as the bony

details of a prenatally-noted anomaly are more evident before further ossification of the vertebra. Neonatal spinal ultrasound performed before extensive laminar ossification has occurred (6-12 weeks) will show major intraspinal anomalies and tethering. Evaluation of the neonatal spine is typically performed with ultrasound and radiography, though MRI sometimes plays a role as well (*54*).

Pediatric spinal dysraphism and associated malformations are accurately diagnosed on an MRI scan. MR myelographic 3D-HASTE and STIR sequences should be a part of the protocol to evaluate spinal dysraphism (*55*). Conventional supine MRI findings may include a low-lying conus medullaris, thickened or fat-infiltrated filum terminale, or lipoma; however, imaging sensitivity and specificity for tethered cord can be low. Prone imaging is found to be a sensitive and specific tool, it may have a role as supportive evidence in the diagnosis of tethered and retethered spinal cord (*56*). New dynamic MRI-based parameters to establish the presence and magnitude of TCS have been defined (*57*).

2.5. Management of SB

Medical management of a child with MMC requires a lifelong multidisciplinary effort including urology, physical orthopedics, and social therapy besides neurosurgery. The initial and probably the most crucial step begins with proper repair of the lesion (*58*). The recommended standard of treatment for open presentations of SB is prenatal surgical repair or postnatal repair within the first few days of life. Prompt postnatal repair has been associated with reduced risk of ventriculoperitoneal (VP) shunt infection, neurogenic bladder (NB), and neurodevelopmental delays (*59*). Early surgical correction of MMC-related spinal deformities improves body balance and quality of life. The dual growing rod technique is safe and effective in cases of moderate neuromuscular spinal deformities at an early age (*60*). The subtraction (decancellation) vertebrectomy technique with preservation of the dural sac is a safe and efficacious technique for correction and stabilization of MMC- kyphosis in young patients. Morbidity is reduced, as compared with excision techniques (*61*).

Symptomatic hydrocephalus is a common condition associated with MMC. Traditionally, hydrocephalus was treated with insertion of a VP shunt. Endoscopic third ventriculostomy (ETV) with choroid plexus cauterization (CPC) and conservative management of relatively stable ventriculomegaly are alternatives to VP shunt placement (*62*). From 1998 to 2014, hydrocephalus treatment has become delayed more and the number of hydrocephalic MMC patients not treated on initial inpatient stay has increased. A meta-analysis demonstrated that shunt malfunction and infection rates do not differ between delayed and simultaneous hydrocephalus treatment (*63*). ETV/CPC is a feasible alternative to ETV and VP shunt in infants with hydrocephalus (*64*).

The Myelomeningocele Study (MOMS trial) was published, demonstrating a decreased need for shunting, a reversal of hindbrain herniation, and better neurologic function in the prenatal repair group compared to postnatal repair with maternal complications and prematurity as a trade-off (*65*). Class I evidence from 1 study and class III evidence from 2 studies suggest that, in comparison to postnatal repair, prenatal surgery for MMC reduces the risk of developing shunt-dependent hydrocephalus. Therefore, prenatal repair of MMC is recommended for those fetuses who meet specific criteria for prenatal surgery to reduce the risk of developing shunt-dependent hydrocephalus (*66*). Despite the confirmed benefits of prenatal surgery, considerable maternal and fetal risk exists compared with postnatal repair. Early gestational age at surgery and development of chorioamniotic membrane separation are risk factors for ruptured membranes (*67*).

Most centers offering open fetal surgery for SB use the MOMS trial criteria to determine eligibility for surgery; some also consider women with a body mass index of 40, those with well controlled insulin dependent diabetes or those who have previously undergone a lower segment cesarean section. In line with the evidence discussed, surgery is typically planned to take place between 23^{+0} and 25^{+6} weeks of gestation (*68*). Maternal obstetric outcomes are superior for fetoscopic SB repair compared to open fetal surgery and avoids the ongoing risk in a future pregnancy. Neonatal and infant benefits appear equivalent (*69*).

Infants with classic cutaneous markers of occult spinal dysraphism, with progressive neurologic, skeletal, and/or urologic findings, present no diagnostic or therapeutic dilemma: they routinely undergo MRI and spinal cord untethering (SCU). Conversely, in asymptomatic patients or those with fixed, minor abnormalities, the risk profile of these occult SB cohorts should be carefully considered before SCU is performed (*70*). Untethering should be performed immediately once the patient shows evidence of symptomatic lumbosacral cord tethering, irrespective of age. Untethering can interrupt the progression of symptoms, but sphincter dysfunction and muscle weakness are more likely to improve or resolve (*71*). However, neurologic recovery with regard to pain and neurologic deficit shows great variation, with improvement rates ranging from 0 to 100%. The causes of tethering, preoperative duration of symptoms, and completeness of untethering could cause the outcomes to vary (*72*). Spine-shortening osteotomy successfully helps to reduce the spinal cord tension without causing direct neural damage. At a minimum, it stabilizes the patients' symptoms and/or helps delay neurological

deterioration for a period of time (*73*). Spine-shortening osteotomy is a safe and effective technique for TCS patients, especially in more challenging cases, such as complex malformations or revision surgery (*74*).

3. Urologic outcomes of SB

Urological manifestations of spinal dysraphism can include increased risks of urinary incontinence, urinary tract infection, urinary calculi, sexual dysfunction, end-stage renal disease, and iatrogenic metabolic disturbances (*75*). Congenital closed spinal anomalies are associated with distortion of the spinal cord, the spinal nerve roots, or both, and can result in neurological abnormalities of the lower limbs and neuropathic bladder dysfunction. All patients with a known or suspected diagnosis of closed SB should have a videourodynamic assessment (*76*).

A study was conducted by Sakakibara *et al.* to assess the urologic and neurologic outcomes in patients diagnosed with SB cystica and occulta. They performed a neurological examination, urinary questionnaire, and urodynamic studies in 28 consecutive patients with urinary symptoms, including 16 with the cystic form, all of whom underwent neonatal surgical management, and 12 with the occult form who did not undergo surgery. Urinary incontinence and enuresis were common at all ages, and large post-micturition residuals and vesicoureteral reflux were not uncommon, particularly in the cystic form. Bladder abnormalities in the cystic and occult forms included detrusor hyperreflexia during filling in 38% and 42%, low compliance detrusor in 81% and 67%, supersensitivity to bethanechol in two (100%) patients with the cystic form and in three of four (75%) with the occult form, and impaired bladder sensation in 25% and 8% in each form, respectively (*77*). Summers *et al.* retrospectively reviewed patients seen at adult dedicated SB clinics at the universities of Utah and Minnesota from April 2011 to April 2012. They identified 65 patients from these clinics with SB. Fifty-five patients (85%) reported a urologic problem at the time of their visit. Urinary incontinence was most common in 34 (52%), followed by recurrent urinary tract infection in 22 (34%), catheterization troubles in 8 (12%), and calculi in 6 (9%). Sixty-three patients (97%) required some sort of intervention. Patients had many active urologic problems and operative management was often needed (*78*).

Bladder dysfunction in SB patients can lead to significant morbidity due to renal insufficiency. Vesicoureteral reflux may occur in up to 40% of children with SB by age 5, and up to 61% of young adults with SB experience urinary incontinence (*79*). In SB, the natural history of the urinary tract in untreated NB and sphincter dysfunction is a progressive deterioration by the age of 3 years in up to 58% of patients. Several reports have shown this deterioration to be directly related to increased intravesical pressure. Without proper management, urinary tract infections and elevated bladder pressures with secondary bladder-wall changes may cause upper urinary tract deterioration (*80*).

4. Urologic Management of SB

Children with MMC can be categorized into high and low- risk groups for secondary damage from a NB based on intravesical pressure. Those with elevated pressure are at risk for hydronephrosis or reflux. Evidence suggests that early management of high pressure protects the bladder from additional damage, reducing the need for augmentation (*81*). Treatment for a child with NB is usually conservative and focuses on achieving safe bladder pressures during storage with reliable emptying, *via* voiding or catheterization. The two most important forms of conservative treatment are CIC and pharmacological treatment of functional disorders. Pharmacologic therapy used for NB are anticholinergic drugs, with the most prescribed antimuscarinic drug as first-line therapy of detrusor overactivity (DO) in children being oxybutynin followed by tolterodine, trospium, solifenacin, and darifenacin (*82*).

In SB patients, it is important to realize that after the closure of the back, pelvic floor behavior can change from paralyzed to overactive in the first 2-3 months of life. That is a reason to delay the first urodynamic study until 2 months after birth. Oxybutynin is best started together with CIC immediately after closure of the back. Repeated injection therapy of the bladder with 300 U of botulinum toxin can be an alternative to antimuscarinic therapy. This therapy effectively suppresses detrusor contractions for 6-9 months. Injections need to be repeated at a 6- to 9-month interval (*83*).

When medical and intravesical options fail to provide satisfactory results, surgical reconstruction may be required to maintain low intravesical storage pressure and achieve treatment goals for urinary continence. Current options for surgical management include incontinent diversion for those who are not candidates for CIC or individualized combinations of augmentation cystoplasty, a bladder outlet procedure, and the creation of a catheterizable channel (*84*). Surgical intervention for patients diagnosed with SB is indicated for those at risk for renal deterioration and/or is considered for children who fail to achieve satisfactory continence with medical management. Traditionally surgery concentrates on the bladder and bladder neck, and creation of catheterizable channels. For those with a hostile bladder, enterocystoplasty remains the gold standard for bladder augmentation, although the use of bowel for augmentation remains suboptimal due to secondary complications, including increased risk of infections, metabolic abnormalities, neoplastic transformation and risk of life-threatening perforation (*11*).

As the child approaches the age of five years, continence becomes an increasing concern. Some patients will be continent between catheterization so no

further intervention is necessary. If maximal medical management remains inadequate, surgical options may be entertained. Adolescence can be a difficult time for these patients. Their medical challenges can take an emotional toll and the social consequences of their mobility, cognitive, and continence can be devastating. The improved care of these patients has resulted in a drastic increase in life expectancy. Although surgical intervention is very prevalent at this age, endoscopic revisions to continent diversions and bladder stones account for a majority of the cases during adulthood (*85*).

Urinary tract calculi remain a large source of morbidity for patients with congenital neuropathic bladder. Patients with NB have a 50% incidence of urinary calculi over 10 years. As with patients without NB, the main strategies to prevent stones typically involve increased fluid intake (*86*). In SB cases, renal function may begin/continue to deteriorate into adulthood, becoming the leading cause of adult death. This is thought to occur because of changes in the adult bladder, with increases in storage pressure. Despite being invalidated in the follow-up of adult SB patient's annual serum creatinine, ultrasound and urodynamics are currently the best tools available (*87*).

The transition from a well-known and trusted pediatric clinic to an unfamiliar adult clinic can be difficult, and the ideal protocol for transition or establishment of care in an adult SB clinic is not clearly defined or standardized. Most adult SB patients continue on anticholinergic medications and CIC. A large percentage of patients require urologic procedures in adulthood (*88*). Potential solutions to improve the urologic care of SB patients suggest additional national provider resources, standardized guidelines, multidisciplinary collaboration, access to care, and an advanced-training pathway to improve the care of adult patients with SB (*89*).

Despite having intact neurological control over erection and ejaculation, other physical limitations and social barriers may hinder sexual intercourse and contribute to infertility in SB men. Urinary incontinence is another source of embarrassment that may contribute to social and performance anxiety when it comes to sexual interactions. Infertility in this population can be caused by problems of sperm transport or defects in spermatogenesis (*90*). In general, adult males with SB have normal sexual desires and an interest in addressing these issues with healthcare providers. 75% of men achieve erections, but maintaining erections is a problem and some may be merely reflexive in nature. Many of these men show marked improvement with sildenafil. In SB patients, the erectile dysfunction and infertility are related to the level of neurological lesion with the best performance status in those with sacral lesions and intact reflexes (*91*).

Deterioration of the bladder is not uncommon in patients with TCS. Although the mechanism of this deterioration has not been elucidated, chronic overdistension of the bladder, is associated with infravesical obstruction (due to detrusor sphincter dyssynergia) and persistent DO. Since TCS-associated urological deterioration can occur at any time during follow-up, urologists should be responsible for examining these patients at regular intervals (*92*). In a study, it was concluded that tethered cord release was beneficial in terms of clinical and urodynamic outcomes. Patients with abnormal urodynamics had a 48% improvement after a tethered cord release. Neurogenic DO seems to respond better with a 59% improvement in urodynamics (*93*). Another study conducted by Abrahamsson *et al.* assessed the urodynamic findings in children with MMC after untethering of the spinal cord. After untethering secondary to MMC, 35% of the patients experienced improved bladder function and 5% deteriorated (*94*). In another study, it was demonstrated that a neurosurgical correction after the appearance of an upper motor neuron sign restored normal neurologic and urinary function in all children; and untethering in children presenting at birth with upper motor neuron symptoms resulted in a poorer outcome (*95*). Table 3 summarizes the available options to manage urological problems in SB patients.

Table 3. Urological management of spina bifida

Medical therapy
Antimuscarinic drugs
Surgical options
Augmentation cystoplasty
Urinary diversion
Bladder neck reconstruction
Catheterizable channels
Other options
Clean intermittent catheterization (CIC)
Intravesical botulinum toxin injections

5. Conclusion

SB is a rare congenital spinal anomaly comprising an open form, which appears in infancy, and an occult form, which appears in late childhood and adulthood. Medical management of a child with MMC requires a multidisciplinary approach including neurosurgeon, urologist, and orthopedist. With urologic management, preservation of kidney function, and continence can be achievable for most SB patients. Children with NB require an intensive lifelong therapy.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Fletcher JM, Brei TJ. Introduction: Spina bifida – a multidisciplinary perspective. Dev Disabil Res Rev. 2010;

16:1-5.
2. Copp AJ, Adzick NS, Chitty LS, Fletcher JM, Holmbeck GN, Shaw GM. Spina bifida. Nat Rev Dis Primers. 2015; 1:15007.
3. Boo NY, Cheah IG, Thong MK; Malaysian National Neonatal Registry. Neural tube defects in Malaysia: data from the Malaysian National Neonatal Registry. J Trop Pediatr. 2013; 59:338-342.
4. Bhide P, Sagoo GS, Moorthie S, Burton H, Kar A. Systematic review of birth prevalence of neural tube defects in India. Birth Defects Res A Clin Mol Teratol. 2013; 97:437-443.
5. Liu J, Zhang L, Li Z, Jin L, Zhang Y, Ye R, Liu J, Ren A. Prevalence and trend of neural tube defects in five counties in Shanxi province of Northern China, 2000 to 2014. Birth Defects Res A Clin Mol Teratol. 2016; 106:267-274.
6. Zaganjor I, Sekkarie A, Tsang BL, Williams J, Razzaghi H, Mulinare J, Sniezek JE, Cannon MJ, Rosenthal J. Describing the prevalence of neural tube defects worldwide: a systematic literature review. PLoS One. 2016; 11:e0151586.
7. Mitchell LE, Adzick NS, Melchionne J, Pasquariello PS, Sutton LN, Whitehead AS. Spina bifida. Lancet. 2004; 364:1885-1895.
8. Bakketun T, Gilhus NE, Rekand T. Myelomeningocele: need for long-time complex follow-up-an observational study. Scoliosis Spinal Disord. 2019; 14:3.
9. Phillips LA, Burton JM, Evans SH. Spina Bifida Management. Curr Probl Pediatr Adolesc Health Care. 2017; 47:173-177.
10. Al-Hazmi HH, Trbay MS, Gomha AB, Elderwy AA, Khatab AJ, Neel KF. Uronephrological outcomes of patients with neural tube defects. Does a spina bifida clinic make a difference? Saudi Med J. 2014; 35(Suppl 1):S64-S67.
11. Snow-Lisy DC, Yerkes EB, Cheng EY. Update on urological management of spina bifida from prenatal diagnosis to adulthood. J Urol. 2015; 194:288-296.
12. Clayton DB, Brock JW 3rd, Joseph DB. Urologic management of spina bifida. Dev Disabil Res Rev. 2010; 16:88-95.
13. Kumar J, Afsal M, Garg A. Imaging spectrum of spinal dysraphism on magnetic resonance: a pictorial review. World J Radiol. 2017; 9:178-190.
14. Dias MS, Partington M. Embryology of myelomeningocele and anencephaly. Neurosurg Focus. 2004; 16:E1.
15. Adzick NS. Fetal myelomeningocele: natural history, pathophysiology, and in-utero intervention. Semin Fetal Neonatal Med. 2010; 15:9-14.
16. Copp AJ, Greene ND. Neural tube defects – disorders of neurulation and related embryonic processes. Wiley Interdiscip Rev Dev Biol. 2013; 2:213-227.
17. Heuer GG, Moldenhauer JS, Scott Adzick N. Prenatal surgery for myelomeningocele: review of the literature and future directions. Childs Nerv Syst. 2017; 33:1149-1155.
18. Mohd-Zin SW, Marwan AI, Abou Chaar MK, Ahmad-Annuar A, Abdul-Aziz NM. Spina bifida: pathogenesis, mechanisms, and genes in mice and humans. Scientifica (Cairo). 2017; 2017:5364827.
19. Juranek J, Salman MS. Anomalous development of brain structure and function in spina bifida myelomeningocele. *Dev Disabil Res Rev.* 2010; 16:23-30.
20. Williams H. A unifying hypothesis for hydrocephalus, Chiari malformation, syringomyelia, anencephaly and spina bifida. Cerebrospinal Fluid Res. 2008; 5:7.
21. McLone DG, Dias MS. The Chiari II malformation: cause and impact. Childs Nerv Syst. 2003; 19:540-550.
22. Elgamal EA. Natural history of hydrocephalus in children with spinal open neural tube defect. Surg Neurol Int. 2012; 3:112.
23. Mayer S, Weisser M, Till H, Gräfe G, Geyer C. Congenital myelomeningocele - do we have to change our management? Cerebrospinal Fluid Res. 2010; 7:17.
24. Hudgins RJ, Gilreath CL. Tethered spinal cord following repair of myelomeningocele. Neurosurg Focus. 2004; 16:E7.
25. Greene ND, Copp AJ. Neural tube defects. Annu Rev Neurosci. 2014; 37:221-242.
26. Eubanks JD, Cheruvu VK. Prevalence of sacral spina bifida occulta and its relationship to age, sex, race, and the sacral table angle: an anatomic, osteologic study of three thousand one hundred specimens. Spine (Phila Pa 1976). 2009; 34:1539-1543.
27. Meling TR, Due-Tønnessen BJ, Lundar T, Helseth E. Okkult spinal dysrafisme [Occult spinal dysraphism]. Tidsskr Nor Laegeforen. 2002; 122:913-916. (in Norwegian)
28. Stoll C, Alembik Y, Dott B. Associated malformations in cases with neural tube defects. Genet Couns. 2007; 18:209-215.
29. Ryznychuk MO, Kryvchanska MI, Lastivka IV, Bulyk RY. Incidence and risk factors of spina bifida in children. Wiad Lek. 2018; 71:339-344.
30. Kondo A, Morota N, Ihara S, Saisu T, Inoue K, Shimokawa S, Fujimaki H, Matsuo K, Shimosuka Y, Watanabe T. Risk factors for the occurrence of spina bifida (a case-control study) and the prevalence rate of spina bifida in Japan. Birth Defects Res A Clin Mol Teratol. 2013; 97:610-615.
31. Agopian AJ, Tinker SC, Lupo PJ, Canfield MA, Mitchell LE; National Birth Defects Prevention Study. Proportion of neural tube defects attributable to known risk factors. Birth Defects Res A Clin Mol Teratol. 2013; 97:42-46.
32. Bergman JE, Otten E, Verheij JB, de Walle HE. Folic acid supplementation influences the distribution of neural tube defect subtypes: A registry-based study. Reprod Toxicol. 2016; 59:96-100.
33. Donnan J, Walsh S, Sikora L, Morrissey A, Collins K, MacDonald D. A systematic review of the risks factors associated with the onset and natural progression of spina bifida. Neurotoxicology. 2017; 61:20-31.
34. Flores AL, Vellozzi C, Valencia D, Sniezek J. Global burden of neural tube defects, risk factors, and prevention. Indian J Community Health. 2014; 26:3-5.
35. Wilde JJ, Petersen JR, Niswander L. Genetic, epigenetic, and environmental contributions to neural tube closure. Annu Rev Genet. 2014; 48:583-611.
36. Copp AJ, Greene ND. Genetics and development of neural tube defects. J Pathol. 2010; 220:217-230.
37. Juriloff DM, Harris MJ. A consideration of the evidence that genetic defects in planar cell polarity contribute to the etiology of human neural tube defects. Birth Defects Res A Clin Mol Teratol. 2012; 94:824-840.
38. Wang L, Xiao Y, Tian T, Jin L, Lei Y, Finnell RH, Ren A. Digenic variants of planar cell polarity genes in human neural tube defect patients. Mol Genet Metab. 2018; 124:94-100.

39. Chen Z, Lei Y, Cao X, Zheng Y, Wang F, Bao Y, Peng R, Finnell RH, Zhang T, Wang H. Genetic analysis of Wnt/PCP genes in neural tube defects. BMC Med Genomics. 2018; 11:38.
40. Amorim MR, Lima MA, Castilla EE, Orioli IM. Non-Latin European descent could be a requirement for association of NTDs and MTHFR variant 677C > T: a meta-analysis. Am J Med Genet A. 2007; 143A:1726-1732.
41. Yan L, Zhao L, Long Y, Zou P, Ji G, Gu A, Zhao P. Association of the maternal MTHFR C677T polymorphism with susceptibility to neural tube defects in offsprings: evidence from 25 case-control studies. PLoS One. 2012; 7:e41689.
42. Yu Y, Wang F, Bao Y, Lu X, Quan L, Lu P. Association between MTHFR gene polymorphism and NTDs in Chinese Han population. Int J Clin Exp Med. 2014; 7:2901-2906.
43. Aneji CN, Northrup H, Au KS. Deep sequencing study of the MTHFR gene to identify variants associated with myelomeningocele. Birth Defects Res A Clin Mol Teratol. 2012; 94:84-90.
44. Martinez CA, Northrup H, Lin JI, Morrison AC, Fletcher JM, Tyerman GH, Au KS. Genetic association study of putative functional single nucleotide polymorphisms of genes in folate metabolism and spina bifida. Am J Obstet Gynecol. 2009; 201:394.e1-11.
45. Greene ND, Stanier P, Copp AJ. Genetics of human neural tube defects. Hum Mol Genet. 2009; 18:R113-R129.
46. Venkataramana NK. Spinal dysraphism. J Pediatr Neurosci. 2011; 6:S31-S40.
47. Dashe JS, Twickler DM, Santos-Ramos R, McIntire DD, Ramus RM. Alpha-fetoprotein detection of neural tube defects and the impact of standard ultrasound. Am J Obstet Gynecol. 2006; 195:1623-1628.
48. Norem CT, Schoen EJ, Walton DL, Krieger RC, O'Keefe J, To TT, Ray GT. Routine ultrasonography compared with maternal serum alpha-fetoprotein for neural tube defect screening. *Obstet Gynecol.* 2005; 106:747-752.
49. Bodin CR, Rasmussen MM, Tabor A, Westbom L, Tiblad E, Ekelund CK, Wulff CB, Vogel I, Petersen OB. Ultrasound in prenatal diagnostics and its impact on the epidemiology of spina bifida in a national cohort from Denmark with a comparison to Sweden. Biomed Res Int. 2018; 2018:9203985.
50. Micu R, Chicea AL, Bratu DG, Nita P, Nemeti G, Chicea R. Ultrasound and magnetic resonance imaging in the prenatal diagnosis of open spina bifida. Med Ultrason. 2018; 20:221-227.
51. Sepulveda W, Wong AE, Sepulveda F, Alcalde JL, Devoto JC, Otayza F. Prenatal diagnosis of spina bifida: from intracranial translucency to intrauterine surgery. Childs Nerv Syst. 2017; 33:1083-1099.
52. Zugazaga Cortazar A, Martín Martinez C, Duran Feliubadalo C, Bella Cueto MR, Serra L. Magnetic resonance imaging in the prenatal diagnosis of neural tube defects. Insights Imaging. 2013; 4:225-237.
53. Milani HJF, Barreto EQS, Chau H, To NH, Moron AF, Meagher S, Da Silva Costa F, Araujo Júnior E. Prenatal diagnosis of closed spina bifida: multicenter case series and review of the literature. J Matern Fetal Neonatal Med. 2020; 33:736-742.
54. Upasani VV, Ketwaroo PD, Estroff JA, Warf BC, Emans JB, Glotzbecker MP. Prenatal diagnosis and assessment of congenital spinal anomalies: Review for prenatal counseling. World J Orthop. 2016; 7:406-417.
55. Mehta DV. Magnetic resonance imaging in paediatric spinal dysraphism with comparative usefulness of various magnetic resonance sequences. J Clin Diagn Res. 2017; 11:TC17-TC22.
56. Stamates MM, Frim DM, Yang CW, Katzman GL, Ali S. Magnetic resonance imaging in the prone position and the diagnosis of tethered spinal cord. J Neurosurg Pediatr. 2018; 21:4-10.
57. Singh S, Behari S, Singh V, Bhaisora KS, Haldar R, Krishna Kumar G, Mishra P, Phadke RV. Dynamic magnetic resonance imaging parameters for objective assessment of the magnitude of tethered cord syndrome in patients with spinal dysraphism. Acta Neurochir (Wien). 2019; 161:147-159.
58. Akalan N. Myelomeningocele (open spina bifida) - surgical management. Adv Tech Stand Neurosurg. 2011; (37):113-141.
59. Radcliff E, Cassell CH, Laditka SB, Thibadeau JK, Correia J, Grosse SD, Kirby RS. Factors associated with the timeliness of postnatal surgical repair of spina bifida. Childs Nerv Syst. 2016; 32:1479-1487.
60. Ryabykh SO, Pavlova OM, Savin DM, Burtsev AV, Gubin AV. Surgical Management of Myelomeningocele-Related Spinal Deformities. World Neurosurg. 2018; 112:e431-e441.
61. Nolden MT, Sarwark JF, Vora A, Grayhack JJ. A kyphectomy technique with reduced perioperative morbidity for myelomeningocele kyphosis. Spine (Phila Pa 1976). 2002; 27:1807-1813.
62. Norkett W, McLone DG, Bowman R. Current management strategies of hydrocephalus in the child with open spina bifida. Top Spinal Cord Inj Rehabil. 2016; 22:241-246.
63. McCarthy DJ, Sheinberg DL, Luther E, McCrea HJ. Myelomeningocele-associated hydrocephalus: nationwide analysis and systematic review. Neurosurg Focus. 2019; 47:E5.
64. Weil AG, Fallah A, Chamiraju P, Ragheb J, Bhatia S. Endoscopic third ventriculostomy and choroid plexus cauterization with a rigid neuroendoscope in infants with hydrocephalus. J Neurosurg Pediatr. 2016; 17:163-173.
65. Moldenhauer JS, Adzick NS. Fetal surgery for myelomeningocele: After the Management of Myelomeningocele Study (MOMS). Semin Fetal Neonatal Med. 2017; 22:360-366.
66. Tamber MS, Flannery AM, McClung-Smith C, Assassi N, Bauer DF, Beier AD, Blount JP, Durham SR, Klimo P Jr, Nikas DC, Rehring P, Tyagi R, Mazzola CA. Congress of neurological surgeons systematic review and evidence-based guideline on the incidence of shunt-dependent hydrocephalus in infants with myelomeningocele after prenatal versus postnatal repair. Neurosurgery. 2019; 85:E405-E408.
67. Johnson MP, Bennett KA, Rand L, Burrows PK, Thom EA, Howell LJ, Farrell JA, Dabrowiak ME, Brock JW 3rd, Farmer DL, Adzick NS; Management of Myelomeningocele Study Investigators. The management of myelomeningocele study: obstetrical outcomes and risk factors for obstetrical complications following prenatal surgery. Am J Obstet Gynecol. 2016; 215:778.e1-778.e9.
68. Sacco A, Ushakov F, Thompson D, Peebles D, Pandya P, De Coppi P, Wimalasundera R, Attilakos G, David AL, Deprest J. Fetal surgery for open spina bifida. Obstet Gynaecol. 2019; 21:271-282.

69. Miller JL, Groves ML, Baschat AA. Fetoscopic spina bifida repair. Minerva Ginecol. 2019; 71:163-170.
70. Tuite GF, Thompson DNP, Austin PF, Bauer SB. Evaluation and management of tethered cord syndrome in occult spinal dysraphism: recommendations from the international children's continence society. Neurourol Urodyn. 2018; 37:890-903.
71. Tseng JH, Kuo MF, Kwang Tu Y, Tseng MY. Outcome of untethering for symptomatic spina bifida occulta with lumbosacral spinal cord tethering in 31 patients: analysis of preoperative prognostic factors. Spine J. 2008; 8:630-638.
72. Nakashima H, Imagama S, Matsui H, Yukawa Y, Sato K, Kanemura T, Kamiya M, Ito K, Matsuyama Y, Ishiguro N, Kato F. Comparative study of untethering and spine-shortening surgery for tethered cord syndrome in adults. Global Spine J. 2016; 6:535-541.
73. Kokubun S, Ozawa H, Aizawa T, Ly NM, Tanaka Y. Spine-shortening osteotomy for patients with tethered cord syndrome caused by lipomyelomeningocele. J Neurosurg Spine. 2011; 15:21-27.
74. Lin W, Xu H, Duan G, Xie J, Chen Y, Jiao B, Lan H. Spine-shortening osteotomy for patients with tethered cord syndrome: a systematic review and meta-analysis. Neurol Res. 2018; 40:340-363.
75. Veenboer PW, de Kort LM, Chrzan RJ, de Jong TP. Urinary considerations for adult patients with spinal dysraphism. Nat Rev Urol. 2015; 12:331-339.
76. Johnston LB, Borzyskowski M. Bladder dysfunction and neurological disability at presentation in closed spina bifida. Arch Dis Child. 1998; 79:33-38.
77. Sakakibara R, Hattori T, Uchiyama T, Kamura K, Yamanishi T. Uroneurological assessment of spina bifida cystica and occulta. Neurourol Urodyn. 2003; 22:328-334.
78. Summers SJ, Elliott S, McAdams S, Oottamasathien S, Brant WO, Presson AP, Fleck J, West J, Myers JB. Urologic problems in spina bifida patients transitioning to adult care. Urology. 2014; 84:440-444.
79. Dorsher PT, McIntosh PM. Neurogenic bladder. Adv Urol. 2012; 2012:816274.
80. Verpoorten C, Buyse GM. The neurogenic bladder: medical treatment. *Pediatr Nephrol.* 2008; 23:717-725.
81. Snodgrass WT, Adams R. Initial urologic management of myelomeningocele. Urol Clin North Am. 2004; 31:427-434.
82. Kroll P. Pharmacotherapy for pediatric neurogenic bladder. Paediatr Drugs. 2017; 19:463-478.
83. de Jong TP, Chrzan R, Klijn AJ, Dik P. Treatment of the neurogenic bladder in spina bifida. Pediatr Nephrol. 2008; 23:889-896.
84. Sturm RM, Cheng EY. The management of the pediatric neurogenic bladder. Curr Bladder Dysfunct Rep. 2016; 11:225-233.
85. Metcalfe PD. Neuropathic bladders: Investigation and treatment through their lifetime. Can Urol Assoc J. 2017; 11:S81-S86.
86. Loftus CJ, Wood HM. Congenital causes of neurogenic bladder and the transition to adult care. Transl Androl Urol. 2016; 5:39-50.
87. Ahmad I, Granitsiotis P. Urological follow-up of adult spina bifida patients. Neurourol Urodyn. 2007; 26:978-980.
88. Liu JS, Greiman A, Casey JT, Mukherjee S, Kielb SJ. A snapshot of the adult spina bifida patient - high incidence of urologic procedures. Cent European J Urol. 2016; 69:72-77.
89. Agrawal S, Slocombe K, Wilson T, Kielb S, Wood HM. Urologic provider experiences in transitioning spina bifida patients from pediatric to adult care. World J Urol. 2019; 37:607-611.
90. Deng N, Thirumavalavan N, Beilan JA, Tatem AJ, Hockenberry MS, Pastuszak AW, Lipshultz LI. Sexual dysfunction and infertility in the male spina bifida patient. Transl Androl Urol. 2018; 7:941-949.
91. Bong GW, Rovner ES. Sexual health in adult men with spina bifida. ScientificWorldJournal. 2007; 7:1466-1469.
92. Park K. Urological evaluation of tethered cord syndrome. J Korean Neurosurg Soc. 2020; 63:358-365.
93. Guerra LA, Pike J, Milks J, Barrowman N, Leonard M. Outcome in patients who underwent tethered cord release for occult spinal dysraphism. J Urol. 2006; 176:1729-1732.
94. Abrahamsson K, Olsson I, Sillén U. Urodynamic findings in children with myelomeningocele after untethering of the spinal cord. J Urol. 2007; 177:331-334.
95. Cornette L, Verpoorten C, Lagae L, Van Calenbergh F, Plets C, Vereecken R, Casaer P. Tethered cord syndrome in occult spinal dysraphism: timing and outcome of surgical release. Neurology. 1998; 50:1761-1765.

Received July 7, 2020; Revised October 4, 2020; Accepted December 12, 2020.

**Address correspondence to:*
Mohamad Abou Chakra, Faculty of Medicine, Department of Urology, Lebanese University, Beirut, Lebanon.
E-mail: mohamedabouchakra@hotmail.com

Released online in J-STAGE as advance publication January 17, 2021.

Review

DOI: 10.5582/irdr.2020.03101

Surveillance and prevalence of fragile X syndrome in Indonesia

Nydia Rena Benita Sihombing[1], Tri Indah Winarni[1], Agustini Utari[1,2], Hans van Bokhoven[3], Randi J Hagerman[4], Sultana MH Faradz[1,*]

[1] Division of Human Genetics, Center for Biomedical Research (CEBIOR), Faculty of Medicine, Diponegoro University/Diponegoro National Hospital, Semarang, Indonesia;
[2] Department of Pediatrics, Faculty of Medicine, Diponegoro University, Semarang, Indonesia;
[3] Department of Human Genetics, Donders Institute for Brain, Cognition, and Behaviour, Radboud University Medical Center, Nijmegen, The Netherlands;
[4] MIND Institute, UC Davis Health, University of California, Davis, California, USA.

SUMMARY Fragile X syndrome (FXS) is the most prevalent inherited cause of intellectual disability (ID) and autism spectrum disorder (ASD). Many studies have been conducted over the years, however, in Indonesia there is relatively less knowledge on the prevalence of FXS. We reviewed all studies involving FXS screening and cascade testing of the high-risk population in Indonesia for two decades, to elucidate the prevalence, as well as explore the presence of genetic clusters of FXS in Indonesia. The prevalence of FXS in the ID population of Indonesia ranged between 0.9-1.9%, while in the ASD population, the percentage was higher (6.15%). A screening and cascade testing conducted in a small village on Java Island showed a high prevalence of 45% in the ID population, suggesting a genetic cluster. The common ancestry of all affected individuals was suggestive of a founder effect in the region. Routine screening and subsequent cascade testing are essential, especially in cases of ID and ASD of unknown etiology in Indonesia.

Keywords fragile X syndrome, intellectual disability, genetic screening, cascade testing

1. Introduction

Fragile X syndrome (FXS) is an X-linked inherited condition that causes developmental problems, including intellectual disability (ID). FXS is caused by the expansion of the cytosine-guanine-guanine (CGG) trinucleotide repeat in the 5' untranslated region (UTR) of the fragile X mental retardation (*FMR1*) gene (OMIM 309550). It has a prevalence of 0.5 to 3 percent in different populations with intellectual disability (ID) and autism spectrum disorder (ASD) (*1*). FXS is characterized by ID and emotional and behavioral disorders, including a short attention span, hyperactivity, tactile defensiveness, and poor eye contact (*2*). Dysmorphic clinical features of FXS include large and prominent ears, macroorchidism during and after puberty, single palmar crease, and hyperextensible joints (*3*). About 50 to 60% of male patients with FXS also have features of autism spectrum disorder (ASD), and FXS is considered to be the most common single gene cause of ASD (*4*). The expansion of CGG trinucleotide repeats is unstable within a specific threshold, with variable length in the normal population. The range of repeats in a normal individual is 5 to 44 repeats. Individuals with 45-54 repeats have variable expansion characteristics and this allele is called an intermediate or 'gray zone' allele, while individuals with 55-200 repeats are classified as premutation carriers. The phenotypes in FXS are associated with more than 200 CGG repeats and methylation, and this range is called the full mutation. Expansion instability usually results from maternal transmission, however, 1 or 2 AGG anchors after every 10 CGG repeats can lead to less frequent expansion to the full mutation when passed on by a mother to the next generation (*5*).

The first cytogenetic analysis of FXS identified the fragile site on the long arm of chromosome X located at Xq27.3, whereby the syndrome was named (*6*). Further molecular analysis for diagnosis of FXS, including a polymerase chain reaction (PCR) based method was introduced after the *FMR1* gene molecular structure was identified in 1991 (*7*). There have been many PCR protocols developed to measure the size of CGG repeats, and PCR is one of the most inexpensive and convenient methods for diagnosis. However, the DNA fragment of expanded repeats in the mid-high premutation range does not amplify well in PCR, so full mutation alleles cannot be detected. Consequently, other methods are

added to differentiate the methylated or unmethylated full mutation alleles (*7,8*). Southern blot analysis using a methylation-sensitive enzyme (*e.g.*, BstZI, EagI, NruI, BssHII) and a non-methylation-sensitive enzyme (*e.g.* EcoRI or HindIII) have been used to detect mid to high premutation and full mutation alleles. Fully expanded alleles are seen as a band significantly larger than 5.2 kb, due to the inability of the enzymes to cut the allele (*9*).

With regard to the accuracy of FXS diagnosis, Southern blotting was established as a gold standard procedure. This method is still considered laborious, time-consuming, and requiring larger amounts of DNA. Novel PCR approaches were studied, and a triplet repeat primed PCR (TP-PCR) was introduced to amplify the CGG-repeat more efficiently. As a result, various kits to identify repeat expansion, as well as to quantify the CGG repeat in the *FMR1* gene are currently employed (*10,11*).

In Indonesia, studies on FXS have been conducted since the early 1990s. The first case of FXS was reported in 1995, when intellectually disabled males attending a special school were screened, identifying the first Javanese family with two affected brothers. Both brothers with FXS had ID with the typical phenotype (*e.g.*, long face, long and prominent ears, and macroorchidism) (*12*). Following a clinical and genetic screening of individuals with ID, other individuals with FXS were found in other institutions or special schools, mostly on Java Island. Cytogenetic analysis and conventional PCR-based screening combined with the fragile X syndrome checklist are still being done for routine FXS diagnosis because of fewer molecular analysis facilities in Indonesia. Southern blot analysis is done only to confirm inconclusive results. We aim to describe the prevalence of FXS in Indonesia, as well as the results of periodic screening and cascade testing in the high-risk population for FXS in Indonesia.

2. Data collection

The data was collected from the previous screening programs from special schools/ institutions, including our recent screening. The previous data of three screenings in 1999, 2012, and 2013 were collected and included from Winarni *et al.* (*13*). The recent screening from institutions and referred patients from the clinic during 2014-2019 were collected for routine cytogenetic analysis, including fragile site detection at chromosome Xq27.3 using G banding technique. The *FMR1* gene was analyzed using PCR-based methods to determine CGG repeat length as previously described (*14,15*). To confirm the diagnosis of FXS, Southern blot analysis was performed (*7,16*). For the most recent screening from an institution, individuals were subjected to *FMR1* molecular analysis using three FastFrax *FMR1* Identification, Sizing, and Methylation Status Kits (The Biofactory Pte Ltd, Singapore) as reported previously (*17*). Other screenings in a remote area of East Indonesia were performed using the chimeric-CGG-primer-based PCR screening method from blood spots samples (*18*). Finally, cascade testing was performed on the family of individuals who were molecularly confirmed as FXS. Informed consent was obtained from all cases, and all studies have been approved by the ethical committee.

3. Prevalence of FXS in Indonesia

In total, six studies have been performed involving screening of high-risk populations, including ID and ASD in Indonesia (Figure 1). The first study conducted by Faradz and colleagues in 1999, yielded 5 out of 262 individuals from an ID population in a special school (1.9%) (*19,20*). The study done by Mundhofir and colleagues in 2012 found nine individuals (1.7%) with FXS from 527 males and females with ID in special schools and institutions in the Central Java province (*16*). The study in an autism population resulted in 4 out of 65 (6.15%) children with FXS, in accordance with a larger prevalence of FXS among individuals with autism (*21*). Blood spot screening in a population of individuals with ID in a remote area of Flores island, East Indonesia found 2 full mutation males and 1 premutation male out of 130 males and 81 females (0.9%), using dried blood spot testing (*18*).

Our latest FXS screening conducted using triplet repeat primed polymerase chain reaction (TP-PCR) in individuals with ID from Central Java province, yielded a prevalence of 1.83% (2 out of 109 individuals) (*17*). A full mutation was found in a male with an IQ of 50 and mild characteristic features of FXS such as, long face, prominent ears, macroorchidism, high arched palate and hyperextensible joints (Figure 2), and a female with mild ID, IQ of 64 without any FXS physical and behavioral characteristics (*17*). Both individuals were not suspected of FXS on physical examination and yielded a lower than threshold score on the Hagerman checklist (*2*). Both affected individuals had siblings with ID, however, follow-up by molecular testing could not be performed, because the parents did not agree on subsequent cascade testing. Thus, other family members were diagnosed based on physical examination and pedigree analysis. Altogether, the prevalence of each screening study is shown in Figure 1.

Upon finding positive results in these studies, a cascade testing was conducted on some individuals. A list of cascade testing results is described in Table 1. Aside from the screening program, some patients were referred to the Diponegoro National Hospital with clinical suspicion of FXS, combined with a family history of ID. Up until 2019, four families were diagnosed molecularly, as shown in Table 2.

Diagnosis of FXS in Indonesia is mostly conducted under university-based studies, done by physicians who lead a research-project and have research-interests in ID through clinical, cytogenetic, and molecular testing.

These studies showed a similar FXS prevalence of 1.7-1.9% of populations with ID of unknown etiology. In addition to our studies, during 2014-2019 there were only four families referred to our research center (Center for Biomedical Research/CEBIOR) by physicians for *FMR1* mutation analysis (Table 2), this might be due to a lack of awareness of the need for genetic testing in those with ID or ASD. The prevalence of FXS is in agreement with the known general prevalence of FXS in diverse populations (*22*), although it is lower than some Asian countries. For example, the prevalence of FXS among boys with ID in Thailand was about 7% (16 of 237 individuals) (*23*), while in Iran, full mutation of *FMR1* was found in 32 of 508 (6.3%) families studied (*24*). These discrepancies are mainly due to the population studied with or without cascade testing, molecular techniques being used, consanguinity, inclusion criteria, and the number of samples.

4. Genetic cluster of FXS in Indonesia

A high rate of FXS cases were found from the first screening in a special school and nearby community of Semin district, of Gunung Kidul regency, province of Yogyakarta in 2000 (20). Screening from a special school revealed 21 out of 47 students (45%) were identified and affected with FXS. After cascade testing was performed, there were 16 nuclear families with 25 affected males,

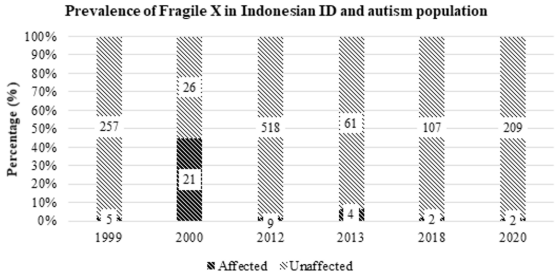


Figure 1. Prevalence of FXS from each screening on high-risk populations in Indonesia. Note the prevalence from the screening of 2000 was higher due to the founder effect, and in 2013 was higher due to the autism population studied.

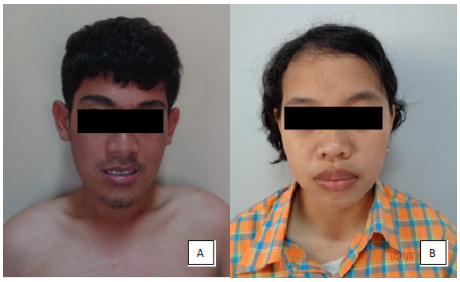


Figure 2. The front facial image of full mutation male (A) and female (B) on the latest FXS screening. FXS clinical stigmata found on the male patient such as long face and prominent ears, while no dysmorphism was observed in the female patient.

Table 1. Cascade testing results on 15 cases from 5 studies

Case no.	No. of nuclear families	Total affected individuals	Total affected males	Total affected females	Total carrier females	Total carrier males	Cascade testing taken from study population
1	1	2	2	0	7	3	Faradz *et al.*, 1999
2	5	5	2	3	6	2	Faradz *et al.*, 1999
3	2	4	4	0	9	3	Faradz *et al.*, 1999
4	2	3	2	1	0	0	Faradz *et al.*, 1999
5	1	1	1	0	1	0	Winarni *et al.*, 2013
6	1	3	2	1	2	1	Winarni *et al.*, 2013
7	2	3	2	1	1	0	Mundhofir *et al.*, 2012
8	3	3	3	0	0	0	Mundhofir *et al.*, 2012
9	1	1	1	0	0	0	Mundhofir *et al.*, 2012
10	2	2	1	1	1	2	Winarni *et al.*, 2013
11	1	3	2	1	1	0	Sihombing *et al.*, 2020
12	1	3	2	1	1	0	Sihombing *et al.*, 2020
13	1	0	0	0	0	1	Utari *et al.*, 2020
14	1	2	1	1	1	0	Utari *et al.*, 2020
15	8	12	5	7	8	1	Utari *et al.*, 2020
Total	32	47	30	17	38	13	

Table 2. Patients referred from the clinic from year 2004 to 2019

Case no.	No. of nuclear families	Total affected individuals	Total affected males	Total affected females	Total carrier females	Total carrier males
A	2	1	1	0	3	0
B	1	3	2	1	1	0
C	1	3	2	1	1	0
D	2	5	5	0	2	2
Total	6	12	10	2	7	2

17 affected females, 27 premutation female carriers, and 6 premutation male carriers, from one large, multi-generational pedigree of the same ancestor, suggesting this founder family was the cause of this high prevalence (*14,20*). The next follow-up in 2005 revealed six additional nuclear families with five affected males, five affected females, five premutation females, and five premutation males (*16*). Our latest follow-up in this region in 2019 revealed no new cases were found using the Hagerman fragile X checklist (*2*).

From approximately 55,000 inhabitants, the estimated number of people with ID in this region was around 400 cases. Out of 42 individuals identified to have a full mutation of *FMR1*, roughly more than 10 percent of cases with ID were FXS, significantly higher than the global prevalence (*20*). A genetic cluster of FXS has been reported in Ricaurte, a district in Colombia, where 1:19 men and 1:46 women carry a full mutation of *FMR1*, while 1:85 men and 1:25 women carry a premutation (*25*). The high prevalence of FXS, the limited geographical area, and the large ancestral pedigree, strongly indicate that Semin district also represents a genetic cluster of FXS.

5. Management of FXS in Indonesia

Management of individuals with FXS-associated disorders comprises long-term health supervision, starting from a young age through adulthood. In addition, to identifying those with *FMR1* premutation-associated disorders such as Fragile-X associated tremor/ataxia syndrome (FXTAS) at a later age, Fragile-X associated primary ovarian insufficiency (FXPOI) among female carriers and the risk of having fragile X-associated neuropsychiatric disorders (FXAND) at all ages (*26,27*). To date, in Indonesia, there are no specific clinical guidelines or recommendations regarding individuals with FXS, premutation disorders or ID in general. Diagnostic workup for FXS is limited, because our center is the only center that performs FXS testing in Indonesia (*16*). Moreover, the cost of diagnostic testing, a multidisciplinary approach for treatment, and long term follow up in order to reach optimum developmental outcomes, besides the need for health care facilities related to comorbidities, are not fully covered by the National Health Insurance (*Jaminan Kesehatan Nasional*/ JKN) plan. These conditions become a challenge for individuals and families who are dealing with FXS.

Genetic counseling is recommended for all family members who: *i*) have a positive result from genetic testing and who may be affected with the full mutation or premutation disorders, *ii*) are at risk of having a FXS child, and *iii*) are at risk of developing premutation-associated disorders. It is important to provide information about the inheritance pattern, risk of having more affected children with FXS or carriers with the premutation. Some women may have the full mutation and these women may not have an ID, but perhaps emotional or learning problems and they need an improved understanding of their health condition (*26*). Cascade testing has revealed that many family members have a full mutation or premutation alleles from each FXS case. Consequently, clinicians or genetic counselors have to improve their understanding of FXS and premutation-associated disorders, the clinical consequences, and the risk of transmitting the disease. If a mother of a child with FXS has a premutation or full mutation allele, she will be able to pass a full mutation to the next offspring, while a carrier father will pass only the premutation allele to all of his daughters (*28*). Moreover, careful consideration should be taken in order to keep the balance of family dynamics, for example on deciding the right time to test and give results to the parents or other family members. There are some cultural aspects to recognize, such as the concern for potential mistreatment of the marital relationship due to carrier status (*28*), or even forced marriage of individuals with FXS due to the social obligation to reproduce offspring (*29*). In Indonesia, aside from cultural belief, a religious aspect also contributes significantly to the attitudes towards illness and decision-making regarding healthcare. Some people would avoid genetic testing and screening due to the belief that the condition is a destiny from God or other superstition related to the carrier who "brings a bad gene" (*30*). The latest follow-up in the area with a high rate of FXS is in the Semin district with no new cases of FXS is suggestive of the acceptance/ understanding of genetic counseling, and our long-term evaluation and follow-up may have an impact on better family planning in this population.

The ongoing research on targeted treatment for FXS has shown some medications that are currently available for clinical use, such as metformin, minocycline, and sertraline. Some promising targeted treatments are still in clinical trials, including cannabidiol, ganaxolone, gaboxadol, arbaclofen, and mavoglurant, among others (*31,32*). Research conducted on children with FXS treated with metformin showed improvements in language development and behavior, such as mood instability and aggressive behavior in most patients (*33*). The availability of testing and screening for FXS in Indonesia, such as in our center will eventually provide access to medication for the patient and family, and will be beneficial for alleviating symptoms, such as anxiety, irritability, and mood disorders, as well as improving language, social communication, and motor skills. Clinical trials remain a challenge in our center due to limited human capacity, the weak regulatory and administrative system, and lack of laboratory facilities for diagnosis, baseline and follow up measurements. However, physicians can take advantage of targeted treatments and prescribe a medication based on the available evidence and a careful consideration of potential risks and benefits for patients.

6. Conclusion

This is the first comprehensive review of FXS in Indonesia. *FMR1* screening is necessary to identify new cases and perform genetic counseling to help families avoid having a recurrence of children with FXS. The diagnosis of individuals with FXS in Indonesia could be significantly improved by more frequent FXS DNA testing of those with ID or ASD by having physicians simply order fragile X DNA testing, since there is improved availability of advanced molecular laboratories and genetic health care professionals such as genetic counselors, clinical geneticists and increasing awareness and collaboration among healthcare providers, government and stakeholders, as well as the community. Regular screening and cascade testing may improve the diagnosis of FXS in previously unknown cases with ID, and further evaluation of other regions of Indonesia, especially outside of Java Island is warranted.

Acknowledgements

We would like to thank all patients and families who have participated in screening and cascade testing.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Willemsen MH, Kleefstra T. Making headway with genetic diagnostics of intellectual disabilities. Clin Genet. 2014; 85:101-110.
2. Hagerman RJ, Amiri K, Cronister A. Fragile X checklist. Am J Med Genet. 1991; 38:283-287.
3. Butler MG, Mangrum T, Gupta R, Singh DN. A 15-item checklist for screening mentally retarded males for the fragile X syndrome. Clin Genet. 1991; 39:347-354.
4. Harris SW, Hessl D, Goodlin-Jones B, Ferranti J, Bacalman S, Barbato I, Tassone F, Hagerman PJ, Herman K, Hagerman RJ. Autism profiles of males with fragile X syndrome. Am J Ment Retard. 2008; 113:427-438.
5. Latham GJ, Coppinger J, Hadd AG, Nolin SL. The role of AGG interruptions in fragile X repeat expansions: a twenty-year perspective. Front Genet. 2014; 5:244.
6. Chudley AE, Hagerman RJ. Fragile X syndrome. J Pediatr. 1987; 110:821-831.
7. Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, Verkerk AJ, Holden JJ, Fenwick RG, Warren ST. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell. 1991; 67:1047-1058.
8. Tassone F. Advanced technologies for the molecular diagnosis of fragile X syndrome. Expert Rev Mol Diagn. 2015; 15:1465-1473.
9. Macpherson J, Sharif A. Practice guidelines for molecular diagnosis of Fragile X Syndrome. Association for Clinical Genetic Science; 2014.
10. Lim GX, Loo YL, Mundhofir FE, Cayami FK, Faradz SM, Rajan-Babu IS, Chong SS, Koh YY, Guan M. Validation of a commercially available screening tool for the rapid identification of CGG trinucleotide repeat expansions in *FMR1*. J Mol Diagn. 2015;17:302-314.
11. Lim GX, Yeo M, Koh YY, Winarni TI, Rajan-Babu IS, Chong SS, Faradz SM, Guan M. Validation of a commercially available test that enables the quantification of the numbers of CGG trinucleotide repeat expansion in *FMR1* gene. PLoS One. 2017; 12:e0173279.
12. Sultana S, Soemantri A, Lam-Po-Tang PRL, Wright F, Lindeman R, Purvis-Smith S. Fragile X mental retardation in an Indonesian family. Medical Journal of Indonesia. 1995; 4:17-23.
13. Winarni TI, Mundhofir FE, Faradz SM. A cohort study of intellectual disability focusing on Fragile X syndrome in Indonesia. Journal of Biomedicine and Translational Research. 2016; 1:2-10.
14. Winarni TI, Mundhofir FE, Ediati A, Belladona M, Nillesen WM, Yntema HG, Hamel BC, Faradz SM, Hagerman RJ. The fragile X-associated tremor ataxia syndrome (FXTAS) in Indonesia. Clin Genet. 2013; 83:263-268.
15. Faradz SM, Pattiiha MZ, Leigh DA, Jenkins M, Leggo J, Buckley MF, Holden JJ. Genetic diversity at the *FMR1* locus in the Indonesian population. Ann Hum Genet. 2000; 64:329-339.
16. Mundhofir FE, Winarni TI, Nillesen W, van Bon BW, Schepens M, Ruiterkamp-Versteeg M, Hamel BC, Yntema HG, Faradz SM. Prevalence of fragile X syndrome in males and females in Indonesia. World J Med Genet. 2012; 2:15-22.
17. Sihombing NRB, Cai S, Wong DPW, Guan M, Chong SS, Faradz SMH, Winarni TI. Repeat expansion and methylation-sensitive triplet-primed polymerase chain reaction for fragile X mental retardation 1 gene screening in institutionalised intellectually disabled individuals. Singapore Med J. 2020; doi: 10.11622/smedj.2020009.
18. Utari A, Basuta K, Winarni TI, Lo J, Morales GM, Faradz SM, Tassone F. Robust screening and cascade testing for fragile X expansions in a large multigenerational family identify many affected individuals: an experience in the remote area of Indonesia. Journal of Intellectual Disability - Diagnosis and Treatment. 2020; 8:9-15.
19. Faradz SM, Buckley M, Lam-Po-Tang, Leigh D, Holden JJ. Molecular screening for fragile X syndrome among Indonesian children with developmental disability. Am J Med Genet. 1999; 83:350-351.
20. Faradz SM, Winarni TI. Focal areas of a high rate of fragile X in Indonesia: a long term follow up. Journal of Biomedicine and Translational Research. 2019; 5:67-68.
21. Winarni T, Utari A, Mundhofir FE, Hagerman RJ, Faradz SM. Fragile X syndrome: clinical, cytogenetic and molecular screening among autism spectrum disorder children in Indonesia. Clin Genet. 2013; 84:577-580.
22. Peprah E. Fragile X syndrome: the FMR1 CGG repeat distribution among world populations. Ann Hum Genet. 2012; 76:178-191.
23. Limprasert P, Ruangdaraganon N, Sura T, Vasiknanonte P, Jinorose U. Molecular screening for fragile X syndrome in Thailand. Southeast Asian J Trop Med Public Health. 1999; 30 Suppl 2:114-118.
24. Pouya AR, Abedini SS, Mansoorian N, Behjati F, Nikzat N, Mohseni M, Nieh SE, Abbasi Moheb L, Darvish H, Monajemi GB, Banihashemi S, Kahrizi K, Ropers HH,

Najmabadi H. Fragile X syndrome screening of families with consanguineous and non-consanguineous parents in the Iranian population. Eur J Med Genet. 2009; 52:170-173.
25. Saldarriaga W, Forero-Forero JV, González-Teshima LY, Fandiño-Losada A, Isaza C, Tovar-Cuevas JR, Silva M, Choudhary NS, Tang HT, Aguilar-Gaxiola S, Hagerman RJ, Tassone F.. Genetic cluster of fragile X syndrome in a Colombian district. J Hum Genet. 2018; 63:509-516.
26. Hersh JH, Saul RA; Committee on Genetics. Health supervision for children with fragile X syndrome. Pediatrics. 2011; 127:994-1006.
27. Winarni TI, Sumekar TA, Wibowo S, Hagerman RJ, Faradz SM. Premutation allele combined with caregiver distress factor increase the risk of depression in fragile X carriers: Indonesia setting. Journal of Intellectual Disability - Diagnosis and Treatment. 2019; 7:200-208.
28. Hagerman RJ, Berry-Kravis E, Hazlett HC, Bailey DB Jr, Moine H, Kooy RF, Tassone F, Gantois I, Sonenberg N, Mandel JL, Hagerman PJ. Fragile X syndrome. Nat Rev Dis Prim. 2017; 3:17065.
29. Pan L, Ye J. Sexuality and marriage of women with intellectual disability in male-squeezed rural China. Sex Disabil. 2012; 30:149-160.
30. Shankar S, Winarni TI, Chonchaiya W, Hoem G, Dy ABC, Sumpaico-Tanchanco L, Gowdra A, Salcedo-Arellano MJ, Saldarriaga W. International perspective on identification and treatment of Fragile X Syndrome and associated disorders. In: Fragile X Syndrome and the Premutation: New Developments and Treatments. Mac Keith Press; 2020. pp. 135-146.
31. Tassanakijpanich N, Cabal-Herrera AM, Salcedo-Arellano MJ, Hagerman RJ. Fragile X syndrome and targeted treatments. Journal of Biomedicine and Translational Research. 2020; 6:23-33.
32. Protic D, Salcedo-Arellano MJ, Dy JB, Potter LA, Hagerman RJ. New targeted treatments for fragile X syndrome. Curr Pediatr Rev. 2019; 15:251-258.
33. Biag HMB, Potter LA, Wilkins V, Afzal S, Rosvall A, Salcedo-Arellano MJ, Rajaratnam A, Manzano-Nunez R, Schneider A, Tassone F, Rivera SM, Hagerman RJ. Metformin treatment in young children with fragile X syndrome. Mol Genet Genomic Med. 2019; 7:e956.

Received September 3, 2020; Revised October 12, 2020; Accepted December 13, 2020.

*Address correspondence to:
Sultana MH Faradz, Center for Biomedical Research (CEBIOR), Faculty of Medicine, Diponegoro University, Jl. Prof. Soedarto SH no. 1, Semarang (50275), Central-Java, Indonesia.
E-mail: sultanafaradz@gmail.com

Released online in J-STAGE as advance publication January 12, 2021.

Mini-Review

DOI: 10.5582/irdr.2020.03123

Different approaches to improve cohort identification using electronic health records: X-linked hypophosphatemia as an example

Jose Jesus Broseta

Department of Nephrology and Renal Transplantation, Hospital Clínic of Barcelona, Barcelona, Spain.

SUMMARY Electronic Health Records (EHRs) represent a source of high value data which is often underutilized because exploiting the information contained therein requires specialized techniques unavailable to the end user *i.e.* the physician or the investigator. Here I describe four simple and practical avenues that will allow the standard EHR end user to identify patient cohorts: the use of diagnostic codes from different international catalogues; a search in reports from complementary tests (*e.g.* radiographs or lab tests) for any result of interest; a free text search; or a drug prescription search in the patient's electronic prescription record. This medical approach is acquiring great importance in the field of rare diseases, and here I demonstrate its application with X-linked hypophosphatemia. The use of these four EHR questioning approaches makes finding a cohort of patients of any condition or disease feasible and manageable, and once each case record is checked, a well-defined cohort can be assembled.

Keywords Cohort identification, electronic health records, patient search, methodological approach, XLH

1. Introduction

Electronic Health Records (EHRs), of common use today, have changed the way clinical information is acquired and documented. They provide quick and economic access to vast amounts of data for clinical and research purposes. However, the exploitation of this information normally uses technical methods, such as natural language processing (NLP), text mining or machine learning techniques which in turn require specialized bioinformatic processes for their implementation (*1*). As a result, this vast amount of data may be inaccessible to the end users, such as clinicians or investigators, who are often looking for patients to recruit for clinical trials or their own observational studies (*2*). The absence of end-user tools greatly diminishes the EHRs' potential as a source of high value information into an underutilized resource that is exacerbated by the number of electronic health record software developers (*3*) and the lack of integration between them (*4*).

In recent years interest has especially grown in the area of rare diseases, which involves thousands of very different diseases that affect a small number of people within the general population (*5*). In Europe, rare diseases are defined as those with a prevalence of less than 1 in every 2,000 people, and within this group a disease is considered to be ultra-rare if it affects 1 in every 50,000 people (*6*). Despite the rising awareness of rare diseases, they still represent a challenge for clinicians, investigators, and public health systems (*7*). The small number of affected patients makes it challenging to investigate the pathophysiological mechanisms and discourages many companies from investing in treatments. In addition, few doctors recognize the heterogeneous signs and symptoms, leading to missed or delayed diagnoses. The length of time for the diagnosis of a rare disease is around 4.8 years, with the patient seeing an average of 7.3 different specialists for case evaluations (*8*). This delay has a serious impact on the patient's quality of life. Earlier identification of the disease is vital, not only for patient follow-up but also for furthering research.

Here, I describe different but compatible approaches that make it possible for a patient cohort to be identified by the standard EHR end user. Whether all or some of these approaches are workable will depend on the EHR developer and the information access provided by the software. For our example, I will apply the four different strategies to the rare disease, X-linked hypophosphatemia.

2. X-linked hypophosphatemia

The estimated incidence and prevalence of X-linked

hypophosphatemia (XLH) are less than 1 in 2,500 and 1 in 20,000, respectively (*9*). Despite the prevalence of this disorder being similar to other known pathologies, and the existence of specialists involved in its diagnosis and treatment, awareness and knowledge about this disease are still relatively low.

XLH has its origin in the mutation of the *PHEX* gene, with a dominant X-linked inherited trait. The mutated *PHEX* translates into an increased serum concentration of fibroblast growth factor 23 (FGF-23), a hormone whose main function is to regulate serum phosphate levels. The decreased clearance of FGF-23 leads to decreased tubular phosphate reabsorption which, together with decreased intestinal absorption of phosphorus, induces hypophosphatemia and ultimately the clinical manifestations of the disease (*10,11*).

The signs and symptoms, which can vary in severity, are evident from the first months of life. The most typical clinical features, which are soon noticeable in childhood, are bowed legs (*genu varum*) and short stature. But there may be other manifestations such as knock-knee (*genu valgum*), frequent fractures, osteoarticular pain, extraosseous calcifications, dental problems or hearing impairment, which in many cases cause a significant functional limitation that negatively impacts the patient's quality of life (*12*). Once suspicion of XLH is raised from the physical examination or the imaging tests, the diagnostic approach should begin by ruling out other causes of rickets, especially deficiencies. Patients with XLH have normal parathyroid hormone (PTH), vitamin D, and calcium in the blood and urine, but serum phosphate levels and tubular phosphate reabsorption are low (*11*).

Conventional treatment is based on oral phosphorus supplements and active vitamin D analogues to maintain alkaline phosphatase within normal range and minimize bone deformities, therefore early diagnosis is essential (*13*). However, these treatments are not without adverse effects, of which the most common are gastrointestinal disorders and the most serious are secondary hyperparathyroidism and nephrocalcinosis (*14*). The development of a new monoclonal antibody has raised new hopes in the management of the disease (*15,16*), as it has shown to stabilize serum phosphate levels and lead to improvements in rickets, skeletal healing, and physical function (*13*). Doctors need to take into account all their current and past patients who could benefit from any therapeutic breakthrough, and those who could participate in new clinical trials and observational studies. This is only possible if XLH patients can be correctly identified.

3. Methods for cohort identification

I demonstrate an EHR search of XLH patients with four easy-to-use data sources.

3.1. Diagnostic codes

One search method available in virtually all institutional systems is searching the diagnostic codes of international catalogues. However, a frequently encountered difficulty is that most catalogues group diseases together with other entities, so extensive case information is needed for correct screening, especially when dealing with rare diseases. This can be achieved using the other methods described below.

The first option is the widely used International Statistical Classification of Diseases and Related Health Problems (ICD) with its different editions: ICD-9, -10 and -11 are the editions most commonly used in hospitals. Many studies have been published using this classification for patient identification (*17-19*).

In ICD-9, XLH is considered a disorder of phosphorus metabolism and the name given is old terminology: vitamin D-resistant rickets, code 275.3. ICD-10 goes a step further in the classification of these disorders of phosphorus metabolism by a separation into four groups: XLH is here coded as E83.30, which encompasses unspecified disorders of phosphorus metabolism. However, ICD-11 made a major change in the classification. It removed XLH from the section of phosphorus metabolism disorders and placed it in the Disorders of vitamin D metabolism or transport, forming its own group, hypophosphatemic rickets, encoded as 5C63.22.

Another widely used classification tool is the Diagnosis-Related Group (DRG), which attempts to classify the diagnosis as reimbursable "products" provided by the hospital, which means that it is designed more for billing than for clinical data analysis (*20*). This system normally places rare diseases, such as XLH, in a general category, classified under "Inborn and other disorders of metabolism", coded as 642.

On the opposite end of the scale is the Systematized Nomenclature of Medicine - Clinical Terms (SNOMED-CT) which is a coded, comprehensive, multilingual, clinical terminology that is becoming a standard in many countries (*21*). Here XLH is given a unique code: 82236004. Another classification database available is the Online Mendelian Inheritance in Man (OMIM), which catalogues all known human genetic disorders; XLH as a hereditary disease has its code: 307800. The summary of the codes for XLH is shown in Figure 1.

3.2. Complementary tests

Another way to find a cohort of patients among the big data provided by the EHRs is to look for diagnostic criteria already discussed in the lab test results or in the imaging reports. Once these findings have been filtered, each case must be reviewed to generate a differential diagnosis that will confirm or rule out the disease.

In the case of XLH, the most effective strategy would be to filter cases of binomial decrease in tubular phosphate reabsorption and hypophosphatemia. This

could raise suspicions of phosphopenic rickets. Even so, we would still have to rule out calcipenic rickets (also known as vitamin D-deficient rickets) by focusing on PTH and vitamin D levels, and Fanconi's syndrome, by looking at urine tests positive for glucosuria, high pH levels and elevated fractional excretion of potassium (*22*). Other approaches that follow this strategy would be to look for the typical radiographic lesions (metaphyseal cupping and flaring and physeal widening and irregularity of the long bones). Additionally, a search for *PHEX* gene mutations should be made in the genetic results database (Figure 2).

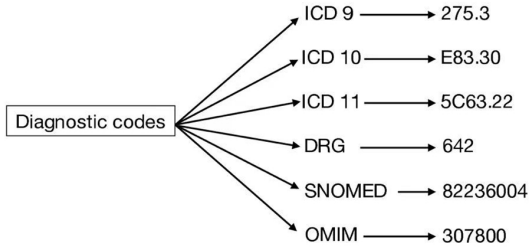


Figure 1. Diagnostic codes for X-linked hypophosphatemia. DRG, diagnosis-related group; ICD, International Statistical Classification of Diseases and Related Health Problems; OMIM, Online Mendelian Inheritance in Man; SNOMED, Systematized Nomenclature of Medicine.

3.3. Free text search

In most EHRs there is the possibility for the clinician to both code their findings in a structured format and also enter information in narrative free text. Unstructured free text searches, when available, can be more efficient than searching for a diagnostic code (*23*). There are different ways to search for free text. One way is to perform a plain text search by entering the different names given to the same disease. In the case of XLH, the different terms include: X-linked hypophosphatemic rickets, X-linked hypophosphatemia, vitamin D-resistant rickets, *etc.* Another alternative would be to search for text in clinical notes, imaging or genetic test reports. This strategy is more far-reaching but less specific than searching for diagnostic codes. It may be time-consuming for very prevalent diseases, but in the case of rare diseases it is best to begin with a long list of potential patients (*24*). Figure 3 shows an example of a free text search for X-linked hypophosphatemia.

An alternative or complementary approach is the use of natural language processing (NLP) to extract important information from text-based documents. This tool has proven to be more accurate in identifying

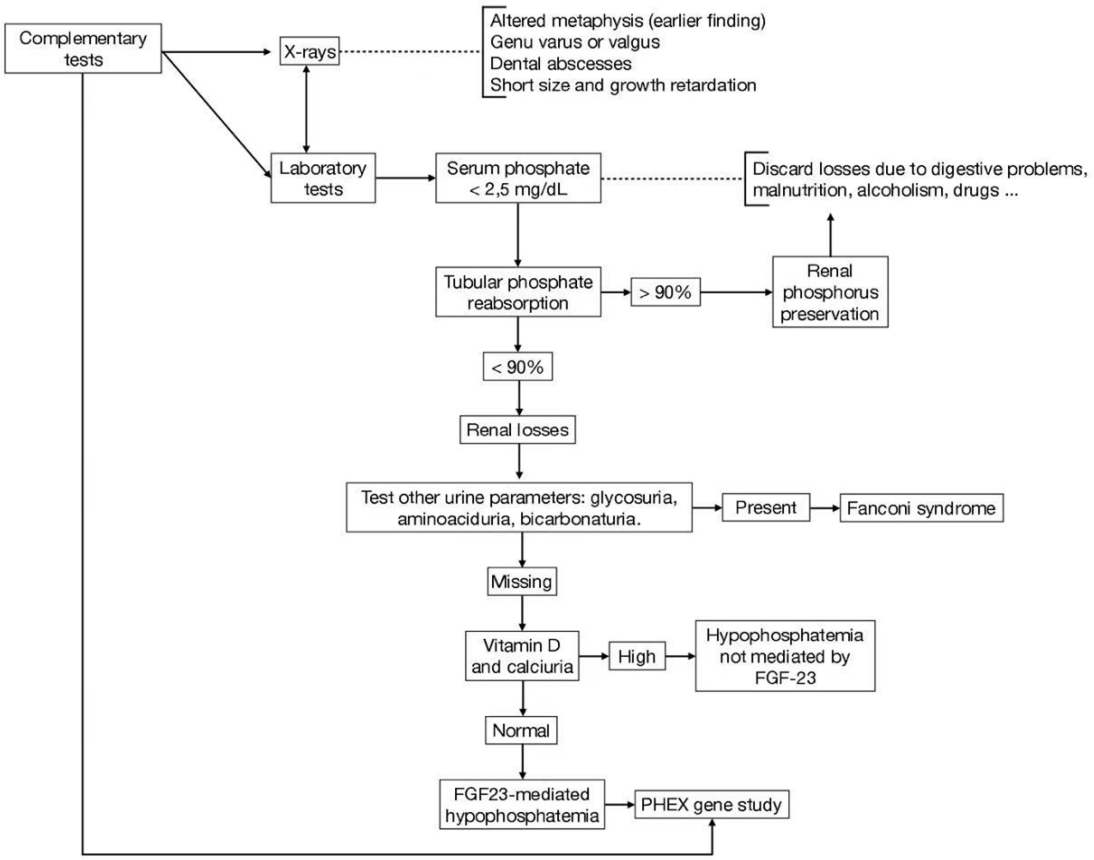


Figure 2. Complementary tests approach for X-linked hypophosphatemia.

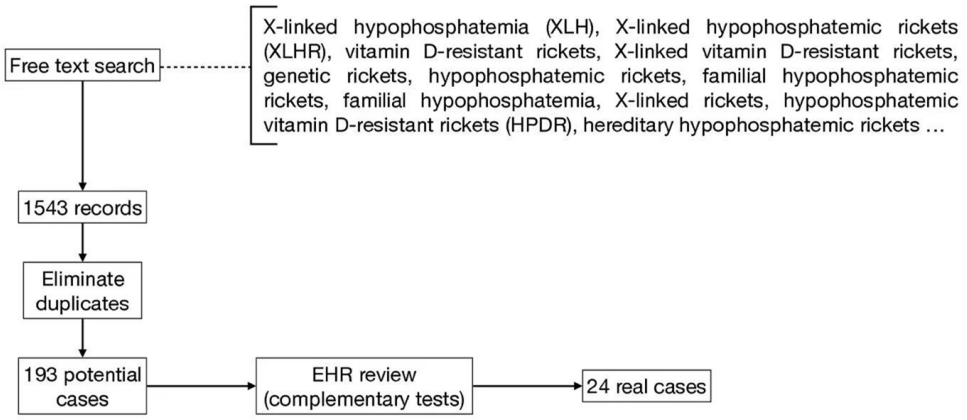


Figure 3. Free text search approach.

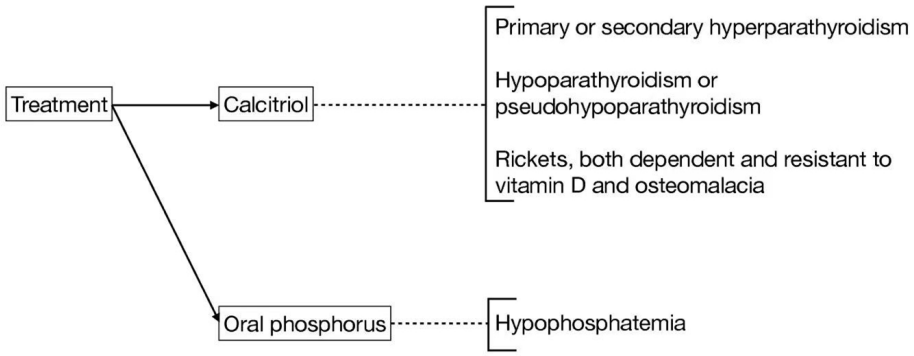


Figure 4. Patients' drug prescription approach.

postoperative complications compared with discharge coding information (*25*). It has also shown advantages in the classification of results of brain tumour magnetic resonance imaging reports in comparison to human expert classification, with excellent accuracy observed for tumour status classification (*26*). Unfortunately, free text searches are rarely exploited (*27,28*).

3.4. Electronic prescriptions

Finally, another way to find cohorts is to search for prescriptions in the patient's electronic medical records. Some drugs indicate specific pathologies, but if that is not the case for the disease being queried, the combination of drugs with several indications may sometimes lead to the disease in question. For example, this method is especially useful in XLH since the classical medical treatment is based on oral supplementation of phosphorus and vitamin D. These two medicinal products, often used separately, are prescribed together in very few situations (Figure 4). This approach can be a useful screening step to then be complemented by the review of each case to confirm the identification. It can be especially valid for those adult patients who received treatment in childhood and were subsequently lost to follow-up (*29*).

4. Conclusion

The identification of cohorts of patients with a certain condition or pathology using "EHR big data" is a growing concern (*1,30*). Sadly, this has become overly technical and out of the reach of doctors or investigators who are not experts in these new technologies (*31*). Therefore, for the EHR data interrogation to successfully satisfy the end user, easier ways are needed to obtain and analyse the information. I have introduced four different ways of finding patients that may be available to the entire medical community. I am aware that each institution with its own EHR software may not have all four of the proposed methods available. However, I am confident that just one or a combination of the methods

would facilitate the task of finding patients with a particular condition. In the case of a rare disease, such as XLH, it is preferable to rely on more sensitive methods, as an eventual "false positive" can always be discarded afterwards. With a well-defined cohort, any investigation or clinical trial can be launched (*32*).

Funding: This article has been commissioned and financially supported by Kyowa Kirin. Neither the author nor the founder have economic or patrimonial interests in the methodological approach explained in this article. The author states that the funder was not involved in the preparation of the manuscript. The author wishes to thank Anabel Herrero, PhD for providing editing assistance. Kyowa Kirin funded this assistance.

Conflict of Interest: JJB reports personal fees and non-financial support from Kyowa Kirin during the conduct of the study.

References

1. Shivade C, Raghavan P, Fosler-Lussier E, Embi PJ, Elhadad N, Johnson SB, Lai AM. A review of approaches to identifying patient phenotype cohorts using electronic health records. J Am Med Inf Assoc. 2014; 21:221-230.
2. Schreiweis B, Trinczek B, Kopcke F, Leusch T, Majeed RW, Wenk J, Bergh B, Ohmann C, Rohrig R, Dugas M, Prokosch HU. Comparison of electronic health record system functionalities to support the patient recruitment process in clinical trials. Int J Med Inf. 2014; 83:860-868.
3. The Office of the National Coordinator for Health Information Technology. Health Care Professional Health IT Developers. 2017; *https://dashboard.healthit.gov/quickstats/pages/FIG-Vendors-of-EHRs-to-Participating-Professionals.php* (accessed August 11, 2020).
4. Jensen PB, Jensen LJ, Brunak S. Mining electronic health records: towards better research applications and clinical care. Nat Rev Genet. 2012; 13:395-405.
5. Schieppati A, Henter JI, Daina E, Aperia A. Why rare diseases are an important medical and social issue. Lancet. 2008; 371:2039-2041.
6. Harari S. Why we should care about ultra-rare disease. Eur Respir Rev. 2016; 25:101-103.
7. Elliott E, Zurynski Y. Rare diseases are a "common" problem for clinicians. Aust Fam Physician. 2015; 44:630-633.
8. Engel P, Bagal S, Broback M, Boice N. Physician and patient perceptions regarding physician training in rare diseases: the need for stronger educational initiatives for physicians. J Rare Disord. 2013; 1:1-15.
9. Beck-Nielsen SS, Brock-Jacobsen B, Gram J, Brixen K, Jensen TK. Incidence and prevalence of nutritional and hereditary rickets in southern Denmark. Eur J Endocrinol. 2009; 160:491-497.
10. Gattineni J, Bates C, Twombley K, Dwarakanath V, Robinson ML, Goetz R, Mohammadi M, Baum M. FGF23 decreases renal NaPi-2a and NaPi-2c expression and induces hypophosphatemia *in vivo* predominantly *via* FGF receptor 1. Am J Physiol Ren Physiol. 2009; 297:F282-291.
11. Pavone V, Testa G, Gioitta Iachino S, Evola FR, Avondo S, Sessa G. Hypophosphatemic rickets: etiology, clinical features and treatment. Eur J Orthop Surg Traumatol. 2015; 25:221-226.
12. Chesher D, Oddy M, Darbar U, Sayal P, Casey A, Ryan A, Sechi A, Simister C, Waters A, Wedatilake Y, Lachmann RH, Murphy E. Outcome of adult patients with X-linked hypophosphatemia caused by *PHEX* gene mutations. J Inherit Metab Dis. 2018; 41:865-876.
13. Lambert AS, Zhukouskaya V, Rothenbuhler A, Linglart A. X-linked hypophosphatemia: Management and treatment prospects. Jt Bone Spine. 2019; 86:731-738.
14. Carpenter TO, Imel EA, Holm IA, Jan de Beur SM, Insogna KL. A clinician's guide to X-linked hypophosphatemia. J Bone Min Res. 2011; 26:1381-1388.
15. Carpenter TO, Whyte MP, Imel EA, Boot AM, Hogler W, Linglart A, Padidela R, Van't Hoff W, Mao M, Chen CY, Skrinar A, Kakkis E, San Martin J, Portale AA. Burosumab therapy in children with X-linked hypophosphatemia. N Engl J Med. 2018; 378:1987-1998.
16. Insogna KL, Briot K, Imel EA, *et al.* A randomized, double-Blind, placebo-controlled, phase 3 trial evaluating the efficacy of burosumab, an anti-FGF23 antibody, in adults with X-linked hypophosphatemia: week 24 primary analysis. J Bone Min Res. 2018; 33:1383-1393.
17. Boyd M, Specks U, Finkielman JD. Accuracy of the ICD-9 code for identification of patients with Wegener's granulomatosis. J Rheumatol. 2010; 37:474.
18. Smith JR, Jones FJS, Fureman BE, Buchhalter JR, Herman ST, Ayub N, McGraw C, Cash SS, Hoch DB, Moura LMVR. Accuracy of ICD-10-CM claims-based definitions for epilepsy and seizure type. Epilepsy Res. 2020; 166:106414.
19. Sun AZ, Shu Y-H, Harrison TN, Hever A, Jacobsen SJ, O'Shaughnessy MM, Sim JJ. Identifying patients with rare disease using electronic health record data: the Kaiser Permanente southern California membranous nephropathy cohort. Perm J. 2020; 24:19.126.
20. Tan JY-A, Senko C, Hughes B, Lwin Z, Bennett R, Power J, Thomson L. Weighted activity unit effect: evaluating the cost of diagnosis-related group coding. Intern Med J. 2020; 50:440-444.
21. Allones JL, Martinez D, Taboada M. Automated mapping of clinical terms into SNOMED-CT. An application to codify procedures in pathology. J Med Syst. 2014; 38:134.
22. González-Lamuño D. Hypophosphataemic rickets: diagnosis algorithm − how not to make a mistake. Adv Ther. 2020; 37:95-104.
23. Kasthurirathne SN, Dixon BE, Gichoya J, Xu H, Xia Y, Mamlin B, Grannis SJ. Toward better public health reporting using existing off the shelf approaches: The value of medical dictionaries in automated cancer detection using plaintext medical data. J Biomed Inf. 2017; 69:160-176.
24. Maguire A, Johnson ME, Denning DW, Ferreira GLC, Cassidy A. Identifying rare diseases using electronic medical records: the example of allergic bronchopulmonary aspergillosis. Pharmacoepidemiol Drug Saf. 2017; 26:785-791.
25. Murff HJ, FitzHenry F, Matheny ME, Gentry N, Kotter KL, Crimin K, Dittus RS, Rosen AK, Elkin PL, Brown SH, Speroff T. Automated identification of postoperative complications within an electronic medical record using natural language processing. JAMA. 2011; 306:848-855.
26. Cheng LT, Zheng J, Savova GK, Erickson BJ. Discerning tumor status from unstructured MRI reports

− completeness of information in existing reports and utility of automated natural language processing. J Digit Imaging. 2010; 23:119-132.
27. Ford E, Nicholson A, Koeling R, Tate A, Carroll J, Axelrod L, Smith HE, Rait G, Davies KA, Petersen I, Williams T, Cassell JA. Optimising the use of electronic health records to estimate the incidence of rheumatoid arthritis in primary care: what information is hidden in free text? BMC Med Res Methodol. 2013; 13:105.
28. Price SJ, Stapley SA, Shephard E, Barraclough K, Hamilton WT. Is omission of free text records a possible source of data loss and bias in Clinical Practice Research Datalink studies? A case-control study. BMJ Open. 2016; 6:e011664-e011664.
29. Seefried L, Smyth M, Keen R, Harvengt P. Burden of disease associated with X-linked hypophosphataemia in adults: a systematic literature review. Osteoporos Int. 2021; 32:7-22.
30. Hemingway H, Asselbergs FW, Danesh J, Dobson R, Maniadakis N, Maggioni A, van Thiel GJM, Cronin M, Brobert G, Vardas P, Anker SD, Grobbee DE, Denaxas S. Big data from electronic health records for early and late translational cardiovascular research: challenges and potential. Eur Hear J. 2018; 39:1481-1495.
31. Hruby GW, Matsoukas K, Cimino JJ, Weng C. Facilitating biomedical researchers' interrogation of electronic health record data: Ideas from outside of biomedical informatics. J Biomed Inf. 2016; 60:376-384.
32. Abrahão MTF, Nobre MRC, Gutierrez MA. A method for cohort selection of cardiovascular disease records from an electronic health record system. Int J Med Inform. 2017; 102:138-149.

Received September 28, 2020; Revised December 19, 2020; Accepted December 25, 2020.

Address correspondence to:
Jose Jesus Broseta, Department of Nephrology and Renal Transplantation, Hospital Clínic of Barcelona, Carrer de Villarroel 170, 08036 Barcelona, Spain.
E-mail: jjbroseta@clinic.cat

Released online in J-STAGE as advance publication January 17, 2021.

Original Article

DOI: 10.5582/irdr.2020.03090

Molecular diagnosis of *SLC26A4*-related hereditary hearing loss in a group of patients from two provinces of Iran

Mahbobeh Koohiyan[1,2], Morteza Hashemzadeh-Chaleshtori[3], Mohammad Amin Tabatabaiefar[1,4,*]

[1] Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran;
[2] Cancer Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran;
[3] Cellular and Molecular Research Center, Basic Health Research Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran;
[4] Pediatric Inherited Diseases Research Center, Research Institute for Primordial Prevention of Noncommunicable Disease, Isfahan University of Medical Sciences, Isfahan, Iran.

SUMMARY The *SLC26A4* gene has been described as the second gene involved in most cases of autosomal recessive non-syndromic hearing loss (ARNSHL), after *GJB2*. Over 500 different *SLC26A4* mutations have been reported, with each ethnic population having its own distinctive mutations. Here, we aimed to determine the frequency and mutation profile of the *SLC26A4* gene from two different provinces (center and west) of Iran. This study included 50 nuclear families with two or more siblings segregating presumed ARNSHL. All affected tested negative for mutations in *GJB2* at the DFNB1 locus and were therefore screened for autozygosity by descent using short tandem repeat polymorphisms (STRPs) of DFNB4. Sanger sequencing was performed to screen the 20 exons of the *SLC26A4* gene for the families linked to this locus. *In silico* analyses were also performed using available software tools. Four out of 25 (16%) and 3 of 25 (12%) studied families of Isfahan and Hamedan provinces, respectively. were linked to DFNB4. Sanger sequencing led to the identification of six different mutations, one of which (c.919-2A>G) was recurrent and accounted for 31% of all mutant alleles. One out of 7 (14.3%) families with mutations were confirmed to be Pendred syndrome (PS). The *SLC26A4* mutations have a high carrying rate in ARNSHL Iranian patients. The identification of a disease causing mutation can be used to establish a genotypic diagnosis and provide important information to the patients and their families.

Keywords autosomal recessive non-syndromic hearing loss, *SLC26A4*, Iran, Pendred syndrome

1. Introduction

Hearing loss (HL) is the most common sensory disorder affecting 2-3 out of 1,000 births (*http://hearing.screening.NHs.UK/nationalprog*); over 70-80% of the etiology are genetic factors. It is estimated that 70% of HL includes non-syndromic forms (NSHL), where the hearing deficit is the only sign. Approximately, 80% of cases in this group follow autosomal recessive inheritance (ARNSHL) (*1*). The autosomal recessive loci are called DFNB followed by a number corresponding to the order that the locus was first described; DFNB1 to DFNB108 have been reported so far (see Hereditary Hearing Loss Homepage at *https://hereditaryhearingloss.org/*).

Mutations in the *SLC26A4* gene have been described for DFNB4 non-syndromic hearing loss (NSHL, MIM # 600791) and Pendred syndrome (PS, MIM # 274600). Pendred syndrome is associated with sensorineural deafness, congenital and severe to profound temporal bone abnormalities, goiter and iodide organification defects. In the absence of thyroid dysfunction, patients are considered to be forms NSHL DFNB4 (*2*).

SLC26A4 (OMIM: 605646) was identified by Everett *et al.* after using positional cloning on chromosome 7q22-31. This gene encodes pendrin, an anion transporter, which is expressed in the kidneys, inner ear, and thyroid (*3*). Pendrin is composed of 780 amino acids and has a molecular weight of 86 k DA. In the inner ear, pendrin was found in the endolymphatic sac and hair cells, where it is involved in pH homeostasis, acting in bicarbonate/chloride exchange. Mutations in the *SLC26A4* gene can affect pendrin activity, causing an imbalance of ions and fluid levels in the inner ear (*4*). Different investigations have suggested that *SLC26A4* mutations are among the most frequent causes of genetic HL in the world populations, including Iranians (*5*). The genetic

etiology of ARNSHL in Iran, has been shown by a number of independent studies with a special focus on DFNB1 (*GJB2*) (*6-12*), the most common cause of HL in the world (*13-15*). In the previous study, we showed that variants in the *GJB2* (NM_004004.5) can explain the etiology of ARNSHL in 22.5% and 20% of patients from Isfahan and Hamedan provinces of Iran, suggesting that *GJB2* gene mutations only represent a part of ARNSHL in the center and west of Iran (*16*). Thus, more studies are necessary to identify other common loci and determine the etiology of ARNSHL based on ethnicity (*17,18*).

In the present study we applied a homozygosity mapping strategy and Sanger sequencing to identify the spectrum and mutation type of the *SLC26A4* gene contribution to ARNSHL. The study cohort included 50 ARNSHL families, negative for *GJB2* mutations, from Isfahan and Hamedan provinces in the center and west of Iran for the first time. This study is part of a larger study, which aims to complete the genetic map of HL in Iran by investigating the pedigrees of families with hearing impaired members in each province. The result of this study should have implications in improved genetic counseling and prevention of HL using pre-implantation genetic diagnosis (PGD) and prenatal diagnosis.

2. Materials and Methods

2.1. Families and phenotype investigation

Fifty consanguineous multiplex families with at least 2 hearing impaired individuals in each family and negative for *GJB2* mutations were recruited for this study. The Ethics Committee of Isfahan University of Medical Sciences approved this project. All family members signed an informed written consent prior to recruitment. They met the following criteria: confirmation of HL by pure tone audiometry (PTA) from 250 to 8000 Hz, autosomal recessive inheritance through pedigree analysis (*3*), existence of three or more affected members within the pedigree. A complete clinical evaluation, including audiology, ophthalmological, and physical examinations were performed to exclude environmental exposures and to determine the presence of syndromic findings in each family.

In two families, both parents were hearing impaired, suggesting the presence of the same identical mutation by descent mutation.

2.2. Genotyping STR markers and linkage analysis

Genomic DNA of patients was extracted from peripheral blood lymphocytes using a standard salting out procedure (*19*). Qualitative and quantitative assessment of genomic DNA was checked using 1.2% agarose gels and a Nanospec cube biophotometer (Nanolytik®, Dusseldorf, Germany).

Linkage analysis was performed using at least four informative Short Tandem Repeat (STR) markers located at or tightly linked to each locus. Primer sequences were obtained from the Probe database. Table 1 summarizes the general characteristics of the markers used in the study. Touchdown PCR was performed in a thermal cycler machine (ASTEC PC-818, Fukuka, Japan) to amplify STR markers. PCR products from family members were genotyped using polyacrylamide gel electrophoresis. We used 12% polyacrylamide (29:1) to detect STR bands. As some STRs were uninformative in some of the families, we had to test more STRs to find at least 4 informative markers for each locus among families to confirm or reject linkage. Two-point and multi-point parametric LOD scores under a recessive model were, respectively, calculated by Superlink version 1.6 and Simwalk version 2.91 (assuming a risk allele frequency of 0.001 and complete penetrance) (easyLINKAGE program package) (*20,21*). Simwalk and Haplopainter software version 029.5 were used to reconstruct and visualize haplotypes, respectively (*22*).

2.3. Mutation detection of SLC26A4

All 20 coding exons (numbered from 2 through 21) of the *SLC26A4* gene were amplified by polymerase chain reaction (PCR) using previously reported primers (*23*). The PCR products were sequenced using a ABI3130 sequencer (Applied Biosystem, Foster City, CA, USA). The resulting sequences were edited and compared against the published NCBI Homo sapiens *SLC26A4* DNA sequence reference assembly (accession NC_000007region: 107301080.107358254).

2.4. Computational analyses

We used Bioinformatics predictive tools including

Table 1. STR markers used and their characteristics

Locus (gene)	STR	Heterozygosity (%)	Size (bp)	Forward Primer	Reverse Primer
DFNB4 (*SLC26A4*)	D7S2420	81	240-290	CCTGTATGGAGGGCAAACTA	AAATAATGACTGAGGCTCAACA
	D7S2496	63	129-141	AACAACAGTCAACCCACAAT	GCTATAACCTCATAACCAAAA
	D7S2459	77	140-152	AAGAAGTGCATTGAGACTCC	CCGCCTTAGTAAAACCC
	D7S2456	78	238-252	CTGGAAATTGACCTGAAACCTT	ACAGGGTCTCTCAATATTA

STR, Short Tandem Repeat.

MutationTaster, PolyPhen and SIFT (*24,25*) to assess possible effects of mutations on the protein structure. Multiple sequence amino acid alignment of the pendrine protein and visualization of conserved amino acids was performed using the ConSurf Server. The American College of Medical Genetics and Genomics (ACMG) guidelines for variant interpretation were used to categorize identified variants (*26*).

2.5. Clinical investigation

Post-test genetic counselling and clinical examination were carried out for all affected members of the linked families, to determine whether the phenotype was related to DFNB4 (NSHL) or PDS (SHL).

Function, structure, and size of the thyroid were evaluated, to determine the presence of goiter, associated with PDS. The levels of thyroid stimulating hormone (*27*), T3 and T4 were measured in all patients by means of Elecsys (Chemiluminescent Immunoassay) to evaluate the function of the thyroid. The size and structure of the thyroid were assessed using a Tc99m thyroid scan. The Perchlorate Discharge Test (PDT) was done in order to confirm the clinical features of PDS. One gram of perchlorate ($KClO_4$) was administered two hours after the administration of 131-iodine (50 mCi). Then, the discharge of iodide was measured. A discharge less than 10% of the incorporated iodide is expected in normal individuals (*28*). All three siblings (family IR-20) also underwent a high-resolution computed tomography (CT) scan of the temporal bone, using a Somatom Sensation 16 (Siemens, Erlangen, Germany), to determine if there were alterations in the cochlea and vestibular aqueduct. When the diameter at the midpoint between the common crus and the external aperture was equal to or greater than 1.5 mm, it was described as Enlarged Vestibular Aqueduct (EVA) (*29*).

3. Results

3.1. Family data and linkage analysis

After excluding mutations in *GJB2*, a total of 50 Iranian families segregating ARNSHL were recruited from Isfahan and Hamedan provinces (25 families from each province) in the center and west of Iran. Among the 50 families (456 individuals), there were 205 patients, with ages ranging from 6 months to 52 years. In 12 studied families, PTA was consistent with severe HL (61-80 dB) and the remaining had profound HL (≥ 80 dB).

The linkage results were confirmed by individually genotyping the family members for the same markers, as well as additional markers. After genotyping of STR markers, 7 out of the 50 families, negative for *GJB2* mutations, showed linkage to DFNB4. 4 out of 25 (16%) and 3 of 25 (12%) ARNSHL families of Isfahan and Hamedan provinces, respectively, were linked to this locus. Two-point and multi-point LOD score values for the seven linked families are shown in Table 2. Our study included two deaf-to-deaf marriages, none of whom were linked to DFNB4.

3.2. SLC26A4 mutation screening

A total of 6 different *SLC26A4* mutations including c.416G>T, c.1334T>G, c.1156A>G, c.1238A>G, c.1295C>T and c.919-2 A>G were detected in 13 out of the 100 (13%) total studied alleles (Table 3). In one

Table 2. LOD score (two-point and multi-point) values for linked families to DFNB4

Number	Family ID	Two-point LOD score	Multi-point LOD score	Severity HL
1	IR-9	3.1	3.2	Severe-profound
2	IR-14	3.8	4.1	Moderate-profound
3	IR-20	2.8	3.2	Severe
4	ISF-5	3	3.3	Severe-profound
5	ISF-6	3.5	3.9	Profound
6	ISF-15	29	3.3	Profound
7	ISF-21	3.2	3.6	Severe-profound

Table 3. Identified *SLC26A4* mutations, their frequencies and *in silico* analyses

Mutations	No (%)		Mutation type	Location	Functional effect		
	Isfahan	Hamedan			PolyPhen Prediction	CADD score	SIFT
c.416 G>T	0	2 (4)	Missense	Exon 05	probably damaging	24.8	Damaging
c.1156 A>G	1	0	Missense	Exon 10	probably damaging	22	Damaging
c.1238 A>G	2 (4)	0	Missense	Exon 10	probably damaging	26.2	Damaging
c.1259 C>T	2 (4)	0	Missense	Exon 10	probably damaging	23.8	Damaging
c.1334 C>T	0	2 (4)	Missense	Exon 11	probably damaging	24.1	Tolerated
c.919-2 A>G	2 (4)	2 (4)	Splice-site	Exon 07	NA	27	NA
Normal	43	44					
Total	50	50					

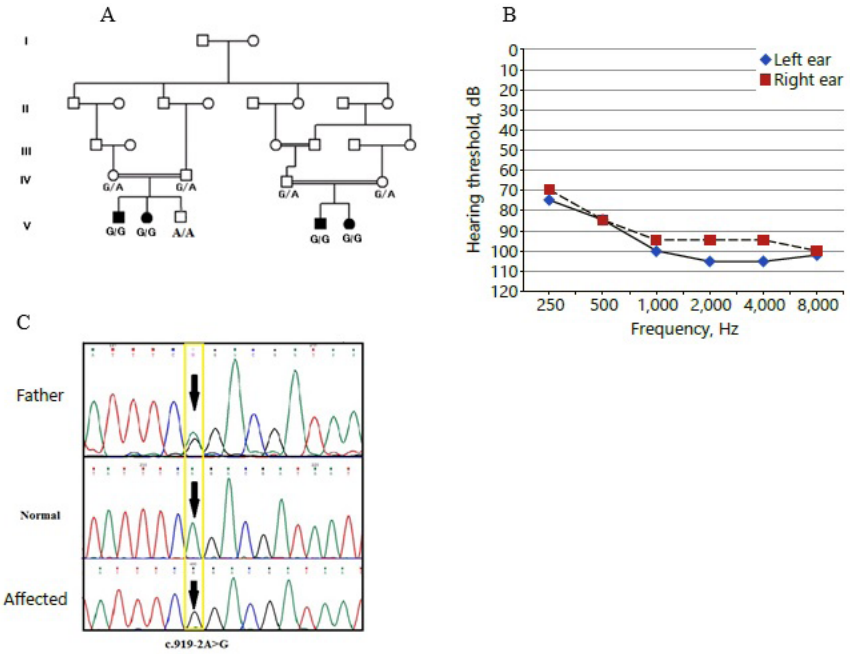


Figure 1. (A) Pedigree and segregation of the c.919-2A>G mutation in Iranian family ISF-5; **(B)** Right and left ear audiograms in the (V: 1) patients from ISF-5 family; **(C)** Sequencing results of the c.919-2A>G mutation.

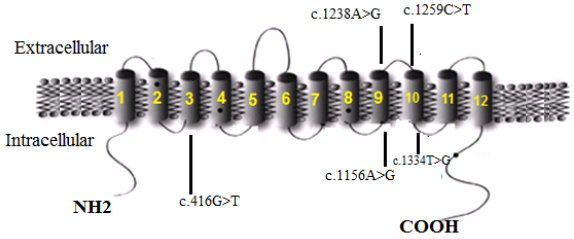


Figure 2. Location of *SLC26A4* mutations at the protein level (The mutation 919-2A>G as an intronic mutation does not change amino acid at the protein level).

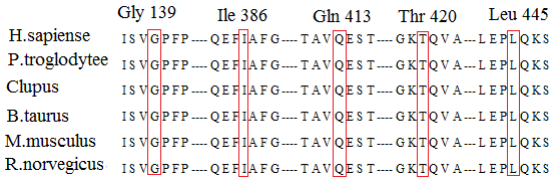


Figure 3. Multiple amino acid alignment of protein homologs was conserved. The identified mutations c.416G>T, c.1334T>G, c.1156A>G, c.1238A>G and c.1295C>T occur at highly conserved positions (Gly 139, Ile 386, Gln 413, Thr 420 and Leu 445) in the pendrin protein.

of the DFNB4 linked families, only one heterozygous mutation was identified, and the remaining five families were homozygotes. Notably, the result of the present study might be a digenic state: interaction with one of the two genes interacting with *SLC26A4*. The missing cause is most likely a non-coding mutation or a large deletion in the coding region. It is certainly possible that a homozygous mutation in another gene could be the underlying cause of HL in this family.

One mutation was a splice site and the others were missense (Table 3). One mutation (c.919-2A>G), was seen in two linked families (IR-9 and ISF-5) from both Isfahan and Hamedan province (Figure 1). All the mutations showed co-segregation with HL in the related family members. Figure 2 shows the distribution of the identified mutations in the schematic structure of pendrin. *In silico* program prediction is shown in Table 3. The ConSurf Server (*http://conseq.tau.ac.il/*) revealed that the causative variants were located at a well conserved site (Figure 3).

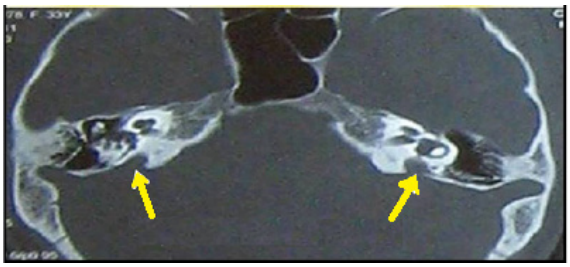


Figure 4. Temporal bone CT scan results of an affected member with EVA.

3.3. Clinical testing results

CT scan showed EVA in all linked families (Figure 4). The levels of thyroid hormones (TSH, T3, and T4) were normal in all linked families, but thyroid scan detected enlarged multinodular goiter in one family (IR-20). There were multiple cold and functioning isoactive nodes on both lobes, which is strong evidence

Table 4. Characterization of Iranian families with *SLC26A4* mutations

Nucleotide Change	Amino Acid Change	Location	Mutation Type	Authors (Year)
c.65-66insT	p.Ser23ValfsAla64	Exon 02	Frameshift	Yazdanpanahi *et al.* (2015)
c.269C>T	p.Ser90Leu	Exon 03	Missense	Sloan-Heggen *et al.* (2015)
c.235C>T	p.Arg79*	Exon 03	Nonsense	Kahrizi *et al.* (2009)
c.347G>T	p.Gly116Val	Exon 04	Missense	Sloan-Heggen *et al.* (2015)
c.416G>T	p.Gly139Val	Exon 05	Missense	Reisi *et al.* (2014)
c.481T>A	p.Phe161Ile	Exon 05	Missense	Sloan-Heggen *et al.* (2015)
c.683C>A	p.Ala228Asp	Exon 06	Missense	Sloan-Heggen *et al.* (2015)
c.716T>A	p.Val239Asp	Exon 06	Missense	Sloan-Heggen *et al.* (2015)
c.863-864insT	p.Leu288PhefsGly3	Exon 07	Frameshift	Yazdanpanahi *et al.* (2015)
c.881-882delAC	p.His294GlnfsGlu35	Exon 07	Frameshift	Yazdanpanahi *et al.* (2015)
c.919-2A>G	–	Intron 07	Splice site	Yazdanpanahi *et al.* (2015)
c.965insA	p.Asn322KfsAla8	Exon 08	Frameshift	Sloan-Heggen *et al.* (2015)
c.959-960insA	p.Gly320LeufsLys20	Exon 08	Frameshift	Sloan-Heggen *et al.* (2015)
c.1001G>T	p.Gly334Val	Exon 08	Missense	Sloan-Heggen *et al.* (2015)
c.1102G>A	p.Gly368Arg	Exon 09	Missense	Sloan-Heggen *et al.* (2015)
c.1156 A>G	p.Ile386Val	Exon 10	Missense	Present study
c.1174A>T	p.Asn392Tyr	Exon 10	Missense	Sloan-Heggen *et al.* (2015)
c.1197delT	p.Cys400Valfsx32	Exon 10	Missense	Kahrizi *et al.* (2009)
c.1226G>A	p.Arg409His	Exon 10	Missense	Kahrizi *et al.* (2009)
c.1229C>T	p.Thr410Met	Exon 10	Missense	Yazdanpanahi *et al.* (2015)
c.1238A>G	p.Gln413Arg	Exon 10	Missense	Yazdanpanahi *et al.* (2015)
c.1259C>T	p.Thr420Ile	Exon 10	Missense	Sloan-Heggen *et al.* (2015)
c.1334T>G	p.Leu445Trp	Exon 11	Missense	Kahrizi *et al.* (2009)
c.1412delT	p.Leu471Argfsx17	Exon 12	Missense	Yazdanpanahi *et al.* (2015)
c.1343C>T	p.Ser448Leu	Exon 12	Missense	Sloan-Heggen *et al.* (2015)
c.1489G>A	p.Gly497Cys	Exon 13	Missense	Yazdanpanahi *et al.* (2015)
c.1517T>G	p.Leu506Arg	Exon 13	Missense	Sloan-Heggen *et al.* (2015)
c.1546C>T	p.Pro516Ser	Exon 13	Missense	Sloan-Heggen *et al.* (2015)
c.1574C>T	p.Pro525Leu	Exon 13	Missense	Sloan-Heggen *et al.* (2015)
c.1614+1G>C	–	Intron 14	Splice site	Sloan-Heggen *et al.* (2015)
c.1717G>T	p.Asp573Tyr	Exon 16	Missense	Sloan-Heggen *et al.* (2015)
c.1790T>C	p.Leu597Ser	Exon 16	Missense	Kahrizi *et al.* (2009)
c.2027T>A	p.Leu676Gln	Exon 19	Missense	Sloan-Heggen *et al.* (2015)
c.2106delG	p.Lys702Asnfsx19	Exon 19	Missense	Yazdanpanahi *et al.* (2015)
c.2162C>T	p.Thr721Met	Exon 19	Missense	Kahrizi *et al.* (2009)
c.2145G>T	p.Lys715Asn	Exon 20	Missense	Kahrizi *et al.* (2009)

supporting that c.1334 C>T is associated with PDS.

4. Discussion

Mutations of *SLC26A4* are a well-known cause of ARNSHL globally. Many studies have been performed to demonstrate the role of *SLC26A4* mutations in ARNSHL among various ethnic cohorts (*30-32*). In a recent multicenter study performed by Sloan-Heggen *et al.*, 37 out of 302 *GJB2*-negative multiplex Iranian families (12.3%) were attributable to variants in the *SLC26A4* gene (*33*), while the frequency of the *SLC26A4* mutations has been reported in Pakistan (*34*) and Turkey to be 5.4%, and 1.8% (*35*) respectively. Mutations of *SLC26A4* are the second most common cause of HL, and have been identified in the sequence encoding all of the transmembrane (TM1-12) segments of this protein (*36*).

The most comprehensive previous study was done on 80 *GJB2*-negative Iranian subjects using linkage analysis and Sanger sequencing showing ~10% ARNSHL families are related to the *SLC26A4* gene and ranked second after DFNB1 (*37*). Our study shows that 4 out of 25 (16%) and 3 of 25 (12%) ARNSHL families of Isfahan and Hamedan provinces, respectively, were linked to DFNB4. The spectrum of *SLC26A4* mutations in our cohort of 50 Iranian families involves mostly missense mutations (70%), which was observed in the other Iranian cohort studies (Table 4) as well as other populations (*38,39*).

c.1156A>G is reported for the first time in Iran. This variant is the adenine to guanine transversion in exon 10 at codon 386 resulting in substitution of isoleucine by valine (P. Ile 386Val). The isoleucine 386 residue is highly conserved among species. Moreover, substitution of residues with a residue, which has different physicochemical properties might result in damaging effects. Isolucine is a nonpolar amino acid with a big hydrophobic structure, while Valine is a small non-charged amino acid. This substitution would affect its contacts with neighboring residues, thereby influencing the folding of the pendrin protein with the mutated residue (*36*).

Three out of 6 (50%) mutations (c.416 G>T, c.919-2A>G and c.1238A>G) were reported in our previous study for the first time in Iran (*23,40*). They may

be unique to the Iranian population, which should be further investigated. Here, exon 10 had the most number of mutations (38.5%). c.1156 A>G, c.1238 A>C and c.1259 C>T mutations are located in exon 10 within the 10th loop putative transmembrane segment of the pendrin protein. Previous studies showed that near 16% of *SLC26A4* mutations are observed within exon 10 (*39*), suggesting that the TM10 region is functionally relevant for pendrin activity (*36*).

In the current study, one out of the 7 (14.3%) families with *SLC26A4* mutations had PS syndrome. In the previous studies in Iran, half of the families with *SLC26A4* mutations were diagnosed with PS (*23*). These results suggest that PS is probably a prevalent syndromic form of HL, which has to be considered in molecular diagnostics of HL in Iran. EVA was a constant feature among all the tested patients in this study.

Unlike *GJB2*, the *SLC26A4* gene has a larger role in Asian than Caucasian populations (*34,41,42*). Studies on NSHL have revealed biallelic *SLC26A4* mutations in 2% to 3.5% of Caucasian patients (*39,43,44*), but in 5.5% to 12.6% of East Asian patients (*42,45,46*). P.Val239Asp was the most common mutation in Turkey (33.3%) and Pakistan (35.6%) while c.919-2A>G and c.1334C>T mutations are more prevalent in East Asia (*34,47*); c.919-2>G has also been found in two of the families in our study. In our patient group, the most frequent mutation is c.919-2A>G, detected in 31% of the mutated alleles. This mutation, which has previously been reported in other ethnic groups, was shown to be a founder mutation in Chinese (*48*) and was reported with a frequency of 31% in a Korean cohort (*49*). However, apart from the present study, no other studies have reported such a high frequency of the c.919-2A>G mutation in Iran (*37*). Notably, c.1238A>G, detected in our previous study, was more common in our population (*23*). The spectrum of mutations in *SLC26A4* in our patients from Isfahan and Hamedan provinces might not be broad, with mutations affecting 3 of 20 exons and exon/intron boundaries. This finding will help design simplified routine DNA testing, targeting the most frequent mutation(s) rather than sequencing all 20 coding exons in patients. Interestingly, the analysis could be started with exon 10, where we found the majority of pathogenic mutations.

The limitation of this study was the sampling of all members of a family. Because both healthy and deaf people had to be sampled, and some members lived in different cities.

5. Conclusion

In the current study, we performed homozygosity mapping analyses and detected 16% and 12% of the studied families to be associated with the *SLC26A4* gene in Isfahan and Hamedan provinces. Our finding will extend the mutation spectrum of the *SLC26A4* gene, and could be applied to prepare a targeted approach to cover the mutations of the *SLC26A4* gene for molecular diagnostics in central and west Iran.

Acknowledgements

This is a partial result of the PhD dissertation of M.K. We would like to thank our patients and their families for participating in this study.

Funding: The Isfahan University of Medical Sciences supported this work (Grant no. 394531, 194068).

Conflict of interest: The authors have no conflict of interest to disclose.

References

1. Morton NE. Genetic epidemiology of hearing impairment. Ann N Y Acad Sci. 1991; 630:16-31.
2. Li XC, Everett LA, Lalwani AK, Desmukh D, Friedman TB, Green ED, Wilcox ER. A mutation in PDS causes non-syndromic recessive deafness. Nat Genet. 1998. 18:215-217.
3. Everett LA, Glaser B, Beck JC, Idol JR, Buchs A, Heyman M, Adawi F, Hazani E, Nassir E, Baxevanis AD, Sheffield VC, Green ED. Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). Nat Genet. 1997; 17:411-422.
4. Albert S, Blons H, Jonard L, *et al. SLC26A4* gene is frequently involved in nonsyndromic hearing impairment with enlarged vestibular aqueduct in Caucasian populations. Eur J Hum Genet. 2006; 14:773-779.
5. Koohiyan M. A systematic review of *SLC26A4* mutations causing hearing loss in the Iranian population. Int J Pediatr Otorhinolaryngol. 2019. 125:1-5.
6. Azadegan-Dehkordi F, Bahrami T, Shirzad M, Karbasi G, Yazdanpanahi N, Farrokhi E, Koohiyan M, Tabatabaiefar MA, Hashemzadeh-Chaleshtori M. Mutations in *GJB2* as major causes of autosomal recessive non-syndromic hearing loss: first report of c.299-300delAT mutation in Kurdish population of Iran. J Audiol Otol. 2019; 23:20-26.
7. Najmabadi H, Nishimura C, Kahrizi K, Riazalhosseini Y, Malekpour M, Daneshi A, Farhadi M, Mohseni M, Mahdieh N, Ebrahimi A, Bazazzadegan N, Naghavi A, Avenarius M, Arzhangi S, Smith RJ. GJB2 mutations: passage through Iran. Am J Med Genet A. 2005; 133A:132-137.
8. Koohiyan M, Ahmadi A, Koohian F, Aghaei S, Amiri B, Hashemzadeh-Chaleshtori M. An update of spectrum and frequency of GJB2 mutations causing hearing loss in the south of Iran: a literature review. Int J Pediatr Otorhinolaryngol. 2019; 119:136-140.
9. Zarepour N, Koohiyan M, Taghipour-Sheshdeh A, Nemati-Zargaran F, Saki N, Mohammadi-Asl J, Tabatabaiefar MA, Hashemzadeh-Chaleshtori M. Identification and clinical implications of a novel MYO15A variant in a consanguineous Iranian family by targeted exome sequencing. Audiol Neurootol. 2019; 24:25-31.

10. Koohiyan M. Next generation sequencing and genetics of hereditary hearing loss in the Iranian population: New insights from a systematic review. Int J Pediatr Otorhinolaryngol. 2020; 129:109756.
11. Koohiyan M. Identification and clinical implications of a novel pathogenic variant in the *GJB2* gene causes autosomal recessive non-syndromic hearing loss in a consanguineous Iranian family. Intractable Rare Dis Res. 2020; 9: 30-34.
12. Koohiyan M, Reiisi S, Azadegan-Dehkordi F, Salehi M, Abtahi H, Hashemzadeh-Chaleshtori M, Noori-Daloii MR, Tabatabaiefar MA. Screening of 10 DFNB loci causing autosomal recessive non-syndromic hearing loss in two Iranian populations negative for *GJB2* mutations. Iran J Public Health. 2019; 48:1704-1713.
13. Lucotte G, Mercier G. Meta-analysis of GJB2 mutation 35delG frequencies in Europe. Genet Test. 2001; 5:149-152.
14. Azadegan-Dehkordi F, Ahmadi R, Koohiyan M, Hashemzadeh-Chaleshtori M. Update of spectrum c.35delG and c.-23+1G>A mutations on the *GJB2* gene in individuals with autosomal recessive nonsyndromic hearing loss. Ann Hum Genet. 2019; 83:1-10.
15. Koohiyan M. Genetics of hereditary hearing loss in the Middle East: a systematic review of the carrier frequency of the GJB2 Mutation (35delG). Audiology and Neurotology, 2019; 24:161-165.
16. Koohiyan M, Hashemzadeh-Chaleshtori M, Salehi M, Abtahi H, Reiisi S, Pourreza MR, Noori-Daloii MR, Tabatabaiefar MA. *GJB2* mutations causing autosomal recessive non-syndromic hearing loss (ARNSHL) in two Iranian populations: Report of two novel variants. Int J Pediatr Otorhinolaryngol. 2018; 107:121-126.
17. Koohiyan M, Hashemzadeh-Chaleshtori M, Salehi M, Abtahi H, Noori-Daloii MR, Tabatabaiefar MA. A novel cadherin 23 variant for hereditary hearing loss reveals additional support for a DFNB12 nonsyndromic phenotype of CDH23. Audiol Neurootol. 2020; 25:258-262.
18. Koohiyan M, Noori-Daloii MR, Hashemzadeh-Chaleshtori M, Salehi M, Abtahi H, Tabatabaiefar MA. A novel pathogenic variant in the CABP2 gene causes severe nonsyndromic hearing loss in a consanguineous Iranian family. Audiol Neurootol. 2019; 24:258-263.
19. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988; 16:1215.
20. Lindner TH, Hoffmann K. easyLINKAGE: a PERL script for easy and automated two-/multi-point linkage analyses. Bioinformatics. 2005; 21:405-407.
21. Fishelson M, Geiger D. Optimizing exact genetic linkage computations. J Comput Biol. 2004; 11:263-275.
22. Thiele H, Nürnberg P. HaploPainter: a tool for drawing pedigrees with complex haplotypes. Bioinformatics. 2005; 21:1730-1732.
23. Yazdanpanahi N, Tabatabaiefar MA, Bagheri N, Azadegan Dehkordi F, Farrokhi E, Hashemzadeh Chaleshtori M. The role and spectrum of *SLC26A4* mutations in Iranian patients with autosomal recessive hereditary deafness. Int J Audiol. 2015; 54:124-130.
24. Schwarz JM, Rödelsperger C, Schuelke M, Seelow D. MutationTaster evaluates disease-causing potential of sequence alterations. Nat Methods. 2010; 7:575-576.
25. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. Nat Methods. 2010; 7:248-249.
26. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015; 17:405-424.
27. Frei K, Ramsebner R, Lucas T, Hamader G, Szuhai K, Weipoltshammer K, Baumgartner WD, Wachtler FJ, Kirschhofer K. GJB2 mutations in hearing impairment: identification of a broad clinical spectrum for improved genetic counseling. Laryngoscope. 2005; 115:461-465.
28. Wolff J. Perchlorate and the thyroid gland. Pharmacol Rev. 1998; 50:89-105.
29. Berrettini S, Forli F, Bogazzi F, Neri E, Salvatori L, Casani AP, Franceschini SS. Large vestibular aqueduct syndrome: audiological, radiological, clinical, and genetic features. Am J Otolaryngol. 2005; 26:363-371.
30. de Moraes VC, dos Santos NZ, Ramos PZ, Svidnicki MC, Castilho AM, Sartorato EL. Molecular analysis of *SLC26A4* gene in patients with nonsyndromic hearing loss and EVA: identification of two novel mutations in Brazilian patients. Int J Pediatr Otorhinolaryngol. 2013; 77:410-413.
31. Azadegan-Dehkordi F, Koohiyan M, Shirzad M, Bahrami T, Yazdanpanahi N, Tabatabaiefar MA, Pourpaknia R, Farrokhi E, Hashemzadeh-Chaleshtori M. Mutation analysis of *GJB2* and *GJB6* genes and screening of nine common dfnb loci in Iranian pedigrees with autosomal recessive nonsyndromic hearing loss. Indian Journal of Otology. 2019; 25:97-102.
32. Tsukada K, Nishio SY, Hattori M, Usami S. Ethnic-specific spectrum of GJB2 and SLC26A4 mutations: their origin and a literature review. Ann Otol Rhinol Laryngol. 2015; 124 Suppl 1:61S-76S.
33. Sloan-Heggen CM, Babanejad M, Beheshtian M, *et al.* Characterising the spectrum of autosomal recessive hereditary hearing loss in Iran. J Med Genet. 2015; 52:823-829.
34. Park HJ, Shaukat S, Liu XZ, *et al.* Origins and frequencies of SLC26A4 (PDS) mutations in east and south Asians: global implications for the epidemiology of deafness. J Med Genet. 2003; 40:242-248.
35. Tekin M, Akçayöz D, Comak E, Boğoçlu G, Duman T, Fitoz S, Ilhan I, Akar N. Screening the *SLC26A4* gene in probands with deafness and goiter (Pendred syndrome) ascertained from a large group of students of the schools for the deaf in Turkey. Clin Genet. 2003; 64:371-374.
36. Bassot C, Minervini G, Leonardi E, Tosatto SC. Mapping pathogenic mutations suggests an innovative structural model for the pendrin (SLC26A4) transmembrane domain. Biochimie. 2017; 132:109-120.
37. Kahrizi K, Mohseni M, Nishimura C, Bazazzadegan N, Fischer SM, Dehghani A, Sayfati M, Taghdiri M, Jamali P, Smith RJ, Azizi F, Najmabadi H. Identification of *SLC26A4* gene mutations in Iranian families with hereditary hearing impairment. Eur J Pediatr. 2009; 168:651-653.
38. Jiang H, Chen J, Shan XJ, Li Y, He JG, Yang BB. Prevalence and range of GJB2 and SLC26A4 mutations in patients with autosomal recessive nonsyndromic hearing loss. Mol Med Rep. 2014; 10:379-386.

39. Rendtorff ND, Schrijver I, Lodahl M, Rodriguez-Paris J, Johnsen T, Hansén EC, Nickelsen LA, Tümer Z, Fagerheim T, Wetke R, Tranebjaerg L. SLC26A4 mutation frequency and spectrum in 109 Danish Pendred syndrome/DFNB4 probands and a report of nine novel mutations. Clin Genet. 2013; 84:388-391.
40. Reiisi S, Sanati MH, Tabatabaiefar MA, Ahmadian S, Reiisi S, Parchami S, Porjafari H, Shahi H, Shavarzi A, Hashemzade Chaleshtori M. The study of *SLC26A4* gene causing autosomal recessive hearing loss by linkage analysis in a cohort of Iranian populations. Int J Mol Cell Med. 2014; 3:176-182.
41. Dai P, Stewart AK, Chebib F, *et al.* Distinct and novel SLC26A4/Pendrin mutations in Chinese and U.S. patients with nonsyndromic hearing loss. Physiol Genomics. 2009; 38:281-290.
42. Miyagawa M, Nishio SY, Usami S; Deafness Gene Study Consortium. Mutation spectrum and genotype-phenotype correlation of hearing loss patients caused by SLC26A4 mutations in the Japanese: a large cohort study. J Hum Genet. 2014; 59:262-268.
43. Dahl HH, Ching TY, Hutchison W, Hou S, Seeto M, Sjahalam-King J. Etiology and audiological outcomes at 3 years for 364 children in Australia. PLoS One. 2013; 8:e59624.
44. Hutchin T, Coy NN, Conlon H, Telford E, Bromelow K, Blaydon D, Taylor G, Coghill E, Brown S, Trembath R, Liu XZ, Bitner-Glindzicz M, Mueller R. Assessment of the genetic causes of recessive childhood non-syndromic deafness in the UK - implications for genetic testing. Clin Genet. 2005; 68:506-512.
45. Yuan Y, Guo W, Tang J, Zhang G, Wang G, Han M, Zhang X, Yang S, He DZ, Dai P. Molecular epidemiology and functional assessment of novel allelic variants of SLC26A4 in non-syndromic hearing loss patients with enlarged vestibular aqueduct in China. PLoS One. 2012; 7:e49984.
46. Guo YF, Liu XW, Guan J, Han MK, Wang DY, Zhao YL, Rao SQ, Wang QJ. GJB2, SLC26A4 and mitochondrial DNA A1555G mutations in prelingual deafness in Northern Chinese subjects. Acta Otolaryngol. 2008; 128:297-303.
47. Lee KY, Choi SY, Bae JW, Kim S, Chung KW, Drayna D, Kim UK, Lee SH. Molecular analysis of the *GJB2*, *GJB6* and *SLC26A4* genes in Korean deafness patients. Int J Pediatr Otorhinolaryngol. 2008; 72:1301-1309.
48. Dai P, Li Q, Huang D, *et al.* SLC26A4 c.919-2A>G varies among Chinese ethnic groups as a cause of hearing loss. Genet Med. 2008; 10:586-592.
49. Park HJ, Lee SJ, Jin HS, Lee JO, Go SH, Jang HS, Moon SK, Lee SC, Chun YM, Lee HK, Choi JY, Jung SC, Griffith AJ, Koo SK. Genetic basis of hearing loss associated with enlarged vestibular aqueducts in Koreans. Clin Genet. 2005; 67:160-165.

Received August 9, 2020; Revised October 22, 2020; Accepted November 17, 2020.

**Address correspondence to:*
Mohammad Amin Tabatabaiefar, Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Hezarjarib St., Isfahan 81746-7346, Iran.
E-mail: tabatabaiefar@gmail.com

Released online in J-STAGE as advance publication November 25, 2020.

Original Article

DOI: 10.5582/irdr.2020.03157

Molecular alteration in the Gap Junction Beta 2 (*GJB2*) gene associated with non-syndromic sensorineural hearing impairment

Smita Hegde[1,3], Rajat Hegde[2,3], Suyamindra S Kulkarni[3], Kusal K Das[2], Pramod B Gai[3,*], Rudregouda Bulgouda[1]

[1] Human genetics laboratory, Department of Anatomy, Shri B. M. Patil Medical College, Hospital and Research centre, BLDE (Deemed to be University), Vijayapura, India;
[2] Laboratory vascular physiology and medicine, Department of Physiology, Shri B. M. Patil Medical College, Hospital and Research centre, BLDE (Deemed to be University), Vijayapura, India;
[3] Karnataka Institute for DNA Research (KIDNAR), Dharwad, India.

SUMMARY Non-syndromic sensory neural hearing defect is one of the genetic diseases inherited from parents to offerings. The autosomal recessive form affects a large population worldwide and has become a major concern in the social and professional lives of many people. There are many factors and genes which are involved in hearing loss but the Gap Junction Beta 2 (*GJB2*) gene which encodes the connexin 26 protein, is a major cause of non-syndromic recessive deafness (NSRD). This study aims to record and analyze *GJB2* gene mutations in the hearing-impaired population of North Karnataka, India. In this study, we included 368 congenitally hearing-impaired children from North Karnataka, India, under 18 years of age. After thorough clinical examinations, patient's history and proper audiological results, peripheral blood samples were collected and subjected to genetic analysis. We recorded that 54.8% of the NSRD cases have an autosomal recessive mutation in the coding region of the *GJB2* gene. The frequency of W24X (25%) mutation was found to be high in the present study population. From this study we can suggest that, identifying this mutation in new-borns definitely helps in the early diagnosis of hearing loss.

Keywords autosomal recessive, non-syndromic recessive deafness, connexin 26, deaf mute, India

1. Introduction

Hearing loss or deafness is one of the most common global health issues, where patients who lose the ability to hear may be permanent or oscillating. Deafness is also one of the most prevalent inherited sensory disorders in most parts of the world (*1*). Approximately 1 in 1,000 new-borns suffer from severe to profound hearing loss. In that > 50% of cases are due to genetic factors (*2*). Environmental factors also play a major role in inducing deafness. For example, congenital hyperbilirubinemia, ototoxic medication, neonatal hypoxia, viral infections, and meningitis are some non-genetic factors inducing congenital hearing loss (*3*). 70-80% of non-syndromic genetic hearing loss is inherited as an autosomal recessive form (DFNB), whereas autosomal dominant forms (DFNA) make up about 10-20% (Dominant forms are designated with the suffix 'A' and recessive forms with suffix 'B') and the X linked form (DFN) accounts for about 1-2% (*4*). DFNB1 gene locus, which is responsible for non-syndromic deafness is present on the 13q11 chromosome locus is most prevalent all over the world (*5*). 13q11 chromosome region includes two major genes viz, connexin 26(*GJB2*) and connexin 30(*GJB6*), which show high involvement in deafness. Connexin 26 (*GJB2*) is the major gene responsible for hearing loss all over the world (*5*). Epidemiological data evaluation of deafness in different populations of the world also revealed that connexin 26 is the single most cause of inherited deafness (*5*). Syndromic forms of deafness which account for more than 500 types can be easily diagnosed whereas non-syndromic forms of deafness can only be resolved by genetic analysis (*6*). The genetic cause of deafness is heterogenous. Until now, more than 100 mapped loci associated with non-syndromic hearing loss have been described (*https://hereditaryhearingloss.org/*). According to the Human Gene Mutation Database (HGMD) the total number of genes involved in hearing loss (HL) is 316, in that 105 genes are related to NSRD. 444 mutations are recorded worldwide specific to the *GJB2* gene (Disease causing mutations-355, non-syndromic HL mutations-51) (*http://*

www.hgmd.cf.ac.uk). W24X mutation in the *GJB2* gene is known to be common in the Indian population. Still, the full spectrum of mutation of this gene occurring in India is not known (*7*).

Connexin is a membrane protein with four transmembrane domains, which are called connexins. These connexin proteins form a hexamer by combining six molecules, called a connexon. A gap junction is formed by the hexamers forming a cell-to-cell channel to the adjacent cells. Small molecules and ions move through this junction to the adjacent cells. Various forms of connexin proteins form different types of hexamers that determine the permeability of different molecules or ions through them (*8*).

2. Materials and Methods

2.1. Subjects

After screening 613 bilateral sensory neural congenital hearing loss children under 18 years of age (median age 12), a total of 368 children (n_{male} = 235, n_{female} = 133) were considered for the study. Children belonging to unrelated families, and having a family history of hearing loss or born to consanguineous marriages were included in the study. Children who had a syndromic hearing loss or were affected by environmental factors and above the age of 18 were excluded from the study. To confirm that the hearing loss occurred because of non-genetic causes (viral and bacterial infections, intake of ototoxic drug during pregnancy, and premature birth), a detailed clinical history and data were collected from each family. After proper physical examination pure tone audiometry (250 to 8,000Hz) was obtained from each child. Clinical samples were collected after obtaining written informed consent from each child. Ethical approval for the study was obtained from the Institutional Ethical Committee of, Shri B. M. Patil Medical College, hospital & research centre, BLDE (Deemed to be University), Vijayapura (Ref no-BLDE(DU)/ IEC/335/2018-19).

2.2. Mutation Analysis

DNA was isolated from peripheral blood using DNeasy blood and tissue kit (QIAGEN, Germany) according to the standard procedures. This was followed by the polymerase chain reaction (New England Biolab, USA). *GJB2* gene (NG_008358) was amplified using specific primers and to check for the proper amplification, PCR products were analyzed by agarose gel electrophoresis. Purified PCR products of the *GJB2* gene were sequenced using forward primer and big dye terminator cycle sequencing kit V3.1 (Applied biosystem, USA) using an ABI 3500 Sanger sequencer. A comparison of the *GJB2* reference sequence to the individual DNA sequence was made to determine the *GJB2* sequence variation.

2.3. Bioinformatics analysis

Pathogenic effects of missense variants were predicted using the following Bioinformatics tools, Protein variation effect analyzer (PROVEN) (*http://provean.jcvi.org/index.php*), Protein Analysis Through Evolutionary Relationships (PANTHER) (*http://www.pantherdb.org*) SNAP2 (*https://www.rostlab.org/services/snap*), Polymorphism Phenotyping2 (PolyPhen-2) (*http://genetics.bwh.harvard.edu/pph2*), Predictor of human Deleterious Single Nucleotide Polymorphisms (PhD-SNP) (*https://snps.biofold.org/phd-snp/phd-snp.html*), SNPs & GO (*https://snps.biofold.org/snps-and-go/snps-and-go.html*) and Deafness Variation Database (DVD) (*https://deafnessvariationdatabase.org/gene/GJB2*). These Bioinformatic tools were used to predict the pathogenic effects of the variant on the functions of the proteins (*9*).

Multiple sequence alignment analyses for connexin 26 protein were performed to find the sequence homology from a common ancestor, which also revealed whether they descended from the same/common ancestor (*10*). Uniport accession numbers from different species were used for the analysis as follows- Xenla *Q7ZYG3*, Mouse *Q009777*, Rat *P21994*, Sheep *P46691*, Macmu *Q8MIT8*, Human *P29033*, Corgo *Q8MHW5*, Canlf *J9NXR*. Homology modelling of the mutated protein was predicted using a Swiss-model server and predicted protein model was visualized, and analyzed on a UCSF chimera program.

3. Results

In this study, we have screened 613 NSRD children from the North Karnataka population. We have included 368 unrelated children with hearing loss (n_{male} = 235, n_{female} = 133) out of that 16.8% (62/368) children had a family history of deafness. Frequency of consanguineous marriage was 42.1% (115/368) in our study. We recorded 18 mutations in exonic and intronic regions of the *GJB2* gene. W24X, and W77X variants were the common mutations identified in this study. A G>A transition at c.71 results in a stop codon at p.24 (W24X) of connexin 26 that produces a truncated protein which is one-tenth the length of the wild type protein and a G>A transition at c.231 results in a stop codon at p.77 (W77X) of connexin 26 protein that also produces truncated protein. Both these premature stop codons result in complete loss of the *GJB2* gene function. W24X (25%) is one of the common mutations observed in the study cohort. 86 (23.3%) deaf children were homozygous and 6 (1.6%) deaf children were heterozygous for this mutation and the W77X mutation was also found to be homozygous (Figure 1).

In our study, we also recorded 3 missense mutations, namely R127H, V153I, and I33T. The frequency of R127H (14.9%) mutation among deaf children was

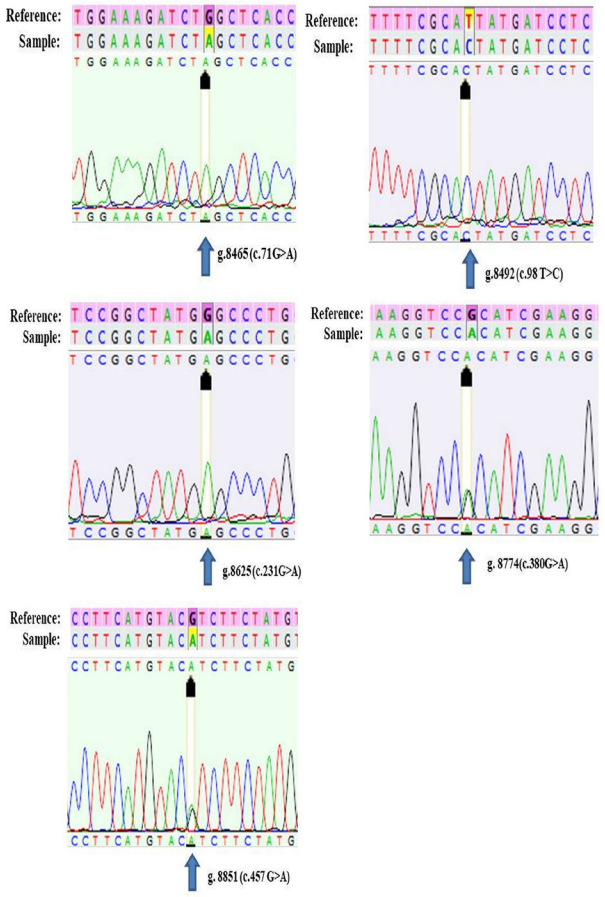


Figure 1. Sequence electropherograms of mutations identified in the present study. W24X (c.71G>A), I33T (c.98T>C), and W77X (c.231G>A) mutations were recorded as homozygous. Whereas R127H (c.380G>A) and V153I (c.457G>A) mutations recorded as heterozygous.

high as compared to the other two identified missense mutations. Four 31-UTR variants were identified (c.84T>C, c.1067G>T, c.1277T>C, c.1152G>A) (Table 1). There were no novel mutations observed in our study population. In addition to these exonic variants, we have also recorded 9 variants in the intronic region of the *GJB2* gene (Table 2), but these variants are unlikely to be causative. The top and middle electropherograms show the homozygous mutations. The position of mutation is indicated by the arrowhead. Heterozygous mutations are in the bottom c.380 G>A and c.451 G>A (Figure 1).

Pathogenicity prediction of the I33T mutation showed the damaging/deleterious effect on the functions of connexin 26 protein. V153I mutations showed a neutral effect, but R127H mutation showed a harmful effect by PhD-SNP, SNP & GO, and SNAP2 (Table 3). DVD database classified these variants, that shows I33T mutation as pathogenic and the other two mutations V153I and R127H as benign (Table 3). Multiple sequence alignment of the connexin 26 protein was analyzed using the Clustal omega. Mutation I33T and R127H were highly conserved over different species and mutation V153I was semi-conserved (Figure 2). The 3D models for mutated Connexin 26 protein (W24X and W77X) were generated using SWISS-MODEL. The superimposed model shows the loss of the majority of the connexin 26 protein sequences from both mutated proteins (Figure 3). Residue W24 and W77 are present in the first transmembrane (S1) domain and second transmembrane (S2) domain of Connexin 26 respectively.

Table 1. List of mutations identified in the coding sequence of *GJB2* gene in the present study

Sl. No.	Nucleotide change	AA change	Frequency	Phenotype	Description and type of effect
1	c.71 G>A	W24X	25%	Congenital profound hearing loss	Stop Gained
2	c.98 T>C	I33T	5.2%	Congenital profound hearing loss	Missense variant
3	c.231 G>A	W77X	4.8%	Congenital severe hearing loss	Stop gained
4	c.380 G>A	R127H	14.9%	Congenital severe hearing loss	Missense
5	c.451 G>A	V153I	4.9%	Congenital profound hearing loss	Missense
6	g.9159T>C	----------	100%	Congenital profound hearing loss	3'-UTR Variant
7	g.10142 G>T	----------	100%	Congenital severe hearing loss	3'-UTR Variant
8	g.10352 T>C	----------	100%	Congenital profound hearing loss	3'-UTR Variant
9	g.10227G>A	----------	5.1%	Congenital severe hearing loss	3'-UTR Variant

Table 2. Mutations were identified in the intronic region of the *GJB2* gene in the present study

Sl. No.	Nucleotide change	Frequency	Phenotype	Description & type of effect
1	g.5985C>T	90%	Congenital profound HI	Regulatory region Variant
2	g.6284A>G	90%	Severe to profound HI	Regulatory region Variant
3	g.6514G>A	14.94%	Congenital profound HI	Regulatory region Variant
4	g.7170A>G	100%	Moderate HI	Regulatory region Variant
5	g.7175G>A	25%	Severe HI	Regulatory region Variant
6	g.8151C>S	4.9%	Severe HI	--------
7	g.8207T>W	14.94%	Congenital profound HI	--------
8	g.8332T>G	30%	Congenital Severe to profound HI	--------
9	g.7111G>R	14%	Congenital profound HI	---------

Table 3. Pathogenicity of the missense variant by insilico tools

Variants	SNAP2	PolyPhen 2	PhD-SNP	SNPS & GO	DVD & CADD	PROVEAN	PANTHER
I33T	Effect Score:55	Possibly damaging Score:0.792	Disease P: 0.548	Disease P: 0.548	Pathogenic 25.2	Deleterious Score: -3.722	Possibly damaging
R127H	Effect Score:1	Benign Score:0.001	Disease P: 0.658	Disease P: 0.589	Benign 23.2	Neutral Score: -0.786	Possibly damaging
V153I	Neutral Score: 74	Benign Score:0.003	Neutral P: 0.149	Neutral P: 0.083	Benign 23.4	Neutral Score: -0.205	Possibly damaging

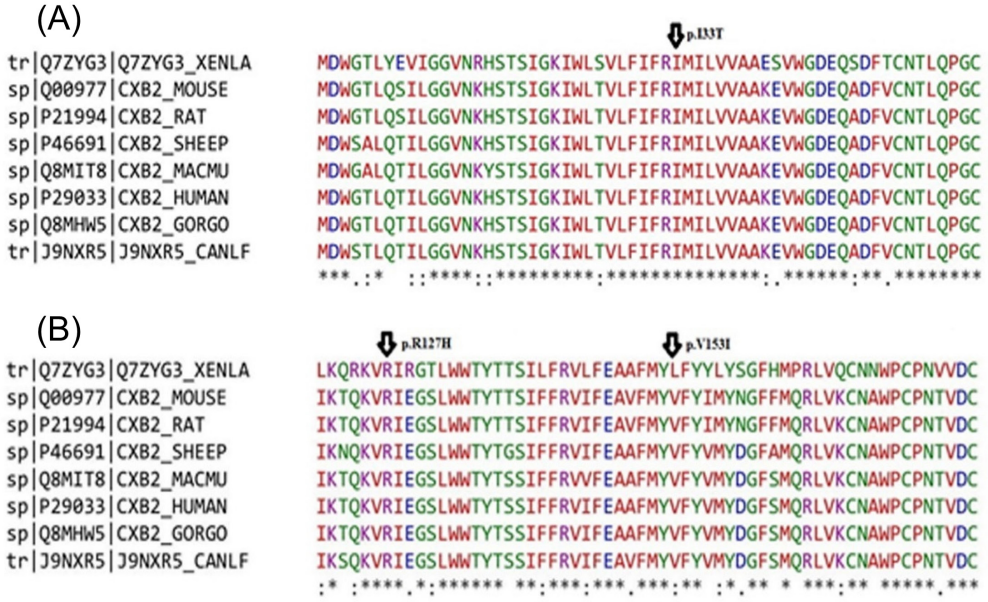


Figure 2. Multiple sequence alignment of connexin 26 protein (A). arrow showing I33T residue conservation (B). first arrow (from left to right) showing the R127H, and second arrow showing the V153I residue conservation.

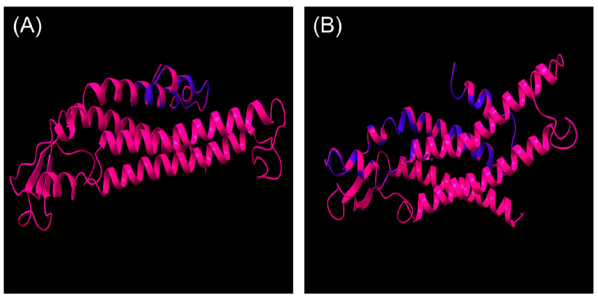


Figure 3. Connexin 26, the 3D structure of the protein. (A), Superimposed connexin 26 protein model with wildtype and truncated protein (Blue- W24 truncated protein, Red- wild type protein); **(B),** superimposed protein structure W77 truncated and wild type protein (Blue- W77 truncated protein, Red- wild type).

4. Discussion

An overview of the mutation spectrum of the connexin 26 gene of 368 cohorts with hearing loss is presented in this study. A worldwide study on the GJB2 gene shows that it is a frequently mutated gene compared to other deafness related gene loci (*11*). W24X (25%), R127H (14.9%), W77X (5.2%) mutation frequencies were found to be higher in our study. 2 missense variations were also recorded in our cohorts viz, I33T (4.9%), and V153I (4.9%). In East Asian lineages, c.235delC is the major mutation that causes hearing loss (*12*). European and African ancestors recorded the 35delG and C.167delT mutations as a causative factor for NSHL (*11*). *GJB2* mutations were found in ~11.5% of deaf families of south Iran and c.35delC was the common mutation identified (*13*). In our study, we didn't find 35delG and C.167delT mutations. Few studies done in India show 35delG and c.71G>A (W24X) as major deafness causing gene mutations (*6,14*). Mutations recorded in the *GJB2*(exon 2) coding region (W24X, W77X), described in this study, have been found previously in a few subjects from Indian subcontinents, including India (*5*). In southern Europe and the United States, congenital deaf cases have biallelic *GJB2* mutations (between 30-35%) (*15-17*). A study on the Chinese Hans population has also recorded 25.65% of Hearing-Impaired patients had biallelic mutations in the *GJB2* gene (*18*). Whereas only 10-20% of congenital deaf cohorts in India have biallelic GJB2 mutations. Therefore, we predict that, there may be additional deafness causing genes that are common in India, other than those already found. We

used the Clustal Omega multiple sequence alignment tool, to analyze evolutionary conservation in each amino acid position. It is observed that R127H, and I33T polymorphisms are highly conserved, and polymorphism V153I is semi conserved (Figure 3). Two different studies transfecting the R127H polymorphism to Hela cells have been conducted. In the first study the R127H mutation acts like a normal connexon 26 protein (*19*). In the second study, it forms the gap junction but there is a reduction in the activity of the gap junction (*5,20*). Bioinformatics analysis on mutations observed in our study also revealed that the R127H polymorphism is non-pathogenic (Table 3). In other Indian populations the R127H mutation strongly suggests that, this is not a causative polymorphism for hearing loss even though its recorded at high frequency. W24X mutation in our cohort shows a heterozygous condition in six subjects with hearing loss and also R127H and V153I polymorphisms were heterozygous in 2 samples. The cause of deafness in those subjects could be because of many factors. The major possibility may be the digenic origin, which is an implication of another connexin gene (*GJB3* or *GJB6*). We have restricted our study to a single gene because of the major involvement of the *GJB2* gene in hearing loss.

Over 200 or more GJB2 gene mutations are recorded on the home page of connexin (*http://davinci.crg.es/deafness*), so it's necessary to sequence the complete coding region of this gene. The frequency of the pathogenic mutation (W24X) in the *GJB2* gene is high in our study population. From this study we can suggest that identifying this mutation in new-borns definitely helps in the early diagnosis of hearing loss. So, implementation of strategies to overcome the disability at an early stage is possible. Using sequencing techniques or restriction analysis, assay mutations can be easily identified. In a country like India, there is a high level of consanguinity and ethnicity and as such these techniques can be easily included, which also help in genetic counselling. This will be beneficial in the early rehabilitation of congenital hearing-impaired children. There is a need for expanding screening in other deafness genes to further resolve *GJB2* mutations. This is the first study to see the effect of genetic variation in deafness in the north Karnataka population. In north Karnataka, out of 12 districts only 4 districts have been included for the genetic analysis of the single targeted gene (GJB2) in hearing impaired children was the major limitation of our study.

Acknowledgements

We thank all the patients and their families for cooperation during the study. The authors also thank all the doctors of the ENT department who participated and helped throughout the study. We thank Karnataka Institute for DNA Research, Dharwad and BLDE (Deemed to be university), and Vijayapura for their constant support throughout the research. We also take this opportunity to express our special thanks to Dr Nandish Kadkol for his help during the study.

Funding: This study was supported by Grants-in-Aid for research from Department of Higher Education, Government of Karnataka (Department of Higher Education, ED 15 UKV 2018, Bangalore, Date 12/13/2018).

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Kelsell DP, Dunlop J, Stevens HP, Lench NJ, Liang JN, Parry G, Mueller RF, Leigh IM. Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. Nature. 1997; 387:80-83.
2. Banjara H, Mungutwar V, Swarnkar N, Patra P. Detection of Connexion 26 GENE (GJB2) Mutations in Cases of Congenital Non Syndromic Deafness. Indian J Otolaryngol Head Neck Surg. 2016; 68:248-253.
3. Pavithra A, Chandru J, Jeffrey JM, Karthikeyen NP, Srisailapathy CRS. Rare compound heterozygosity involving dominant and recessive mutations of GJB2 gene in an assortative mating hearing impaired Indian family. Eur Arch Otorhinolaryngol. 2017; 274:119-125.
4. Duman D, Tekin M. Autosomal recessive nonsyndromic deafness genes: A review. Front Biosci (Landmark Ed). 2012; 17:2213-2236.
5. Ramshankar M, Girirajan S, Dagan O, Shankar HMR, Jalvi R, Rangasayee R, Avraham KB, Anand A. Contribution of connexin26 (GJB2) mutations and founder effcet to non-syndromic hearing loss in India. J Med Genet. 2003; 40:e68.
6. Ghosh M, Vijaya R, Kabra M. Genetics of deafness in India. Indian J Pediatr. 2004; 71:531-533.
7. Mani RS, Ganapathy A, Jalvi R, Srikumari Srisailapathy CR, Malhotra V, Chadha S, Agarwal A, Ramesh A, Rangasayee RR, Anand A. Functional consequences of novel connexin 26 mutations associated with hereditary hearing loss. Eur J Hum Genet. 2009; 17:502-509.
8. Kemperman MH, Hoefsloot LH, Cremers CWRJ. Hearing loss and connexin 26. J R Soc Med. 2002; 95:171-177.
9. Yilmaz A. Bioinformatic analysis of GJB2 gene missense mutations. Cell Biochem Biophys. 2015; 71:1623-1642.
10. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. 2003; 31:3497-3500.
11. Tariq H, Zaigham K, Kousar S, Azhar A. Genetic contribution of GJB2 gene to hearing impairment in Pakistan. Advancements in Life Sciences. 2019; 7:38-43.
12. Kudo T, Ikeda K, Kure S, Matsubara Y, Oshima T, Watanabe KI, Kawase T, Narisawa K, Takasaka T. Novel mutations in the connexin 26 gene (GJB2) responsible for childhood deafness in the Japanese population. Am J Med Genet. 2000; 90:141-145.
13. Koohiyan M, Azadegan-Dehkordi F, Koohian F, Hashemzadeh-Chaleshtori M. Genetics of hearing loss in north Iran population: An update of spectrum and frequency of GJB2 mutations. J Audiol Otol. 2019; 23:175-180.

14. Mishra S, Pandey H, Srivastava P, Mandal K, Phadke SR. Connexin 26 (GJB2) mutations associated with non-syndromic hearing loss (NSHL). Indian J Pediatr. 2018; 85:1061-1066.
15. Lench N, Houseman M, Newton V, Van Camp G, Mueller R. Connexin-26 mutations in sporadic non-syndromal sensorineural deafness. Lancet. 1998; 351:415.
16. Denoyelle F, Weil D, Maw MA, *et al*. Prelingual deafness: High prevalence of a 30delG mutation in the connexin 26 gene. Hum Mol Genet. 1997; 6:2173-2177.
17. Kelley PM, Harris DJ, Comer BC, Askew JW, Fowler T, Smith SD, Kimberling WJ. Novel mutations in the connexin 26 gene (GJB2) that cause autosomal recessive (DFNB1) hearing loss. Am J Hum Genet. 1998; 62:792-799.
18. Yu X, Lin Y, Xu J, Che T, Li L, Yang T, Wu H. Molecular epidemiology of Chinese Han deaf patients with bi-allelic and mono-allelic GJB2 mutations. Orphanet J Rare Dis. 2020; 15:29.
19. Thönnissen E, Rabionet R, Arbonès ML, Estivill X, Willecke K, Ott T. Human connexin26 (GJB2) deafness mutations affect the function of gap junction channels at different levels of protein expresion. Hum Genet. 2002; 111:190-197.
20. D'Andrea P, Veronesi V, Bicego M, Melchionda S, Zelante L, Di Iorio E, Bruzzone R, Gasparini P. Hearing loss: Frequency and functional studies of the most common connexin26 alleles. Biochem Biophys Res Commun. 2002; 296:685-691.

Received December 16, 2020; Revised January 15, 2021; Accepted January 27, 2021.

**Address correspondence to:*
Pramod B Gai, Karnataka Institute for DNA Research Dharwad 580003, India.
E-mail: pramodbgai@gmail.com

Released online in J-STAGE as advance publication February 5, 2021.

Original Article

DOI: 10.5582/irdr.2020.03085

Clinical correlation and antimicrobial susceptibility pattern of *Chryseobacterium* spp.: A three year prospective study

Vishwanath Singh Yadav, Bimal Ku Das*, Sarita Mohapatra, M Nizam Ahmed, Hitender Gautam, Arti Kapil, Seema Sood, Benu Dhawan, Rama Chaudhry

Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India.

SUMMARY *Chryseobacterium species* are widely distributed in the environment. They are rarely found in hospital settings causing nosocomial infections. Limited data is available regarding their epidemiology, clinical significance and antimicrobial susceptibility patterns. This study was aimed to identify different species of *Chryseobacterium* using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and to correlate clinically with antimicrobial susceptibility patterns in a tertiary care hospital in north India. We also performed phenotypic tests, which may be useful to differentiate this bacterium from other non-fermenters. A total of 20 isolates of *Chryseobacterium* spp. were identified over a period of 3 years. *Chryseobacterium indologenes* (18/20) was the most common species isolated followed by *Chryseobacterium gleum* (2/20) from various clinical samples. Antimicrobial susceptibility testing (AST) was performed. Susceptibility to rifampicin was observed at a maximum (75%) followed by piperacillin-tazobactum (45%). Susceptibility against imipenem, meropenem, cotrimoxazole and cefoperazone-sulbactum were observed approximately 33%. Amikacin, cefotaxime and ceftazidime showed least susceptibility results. Further clinical correlation was established.

Keywords *Chryseobacterium indologenes*, *Chryseobacterium gleum*, nosocomial infections, MALDI-TOF MS

1. Introduction

Chryseobacterium species are among the emerging pathogens causing infections in humans. They are rarely isolated from clinical samples (*1*). There are more than 100 species of *Chryseobacterium* identified until now (*2*). However, only *Chryseobacterium indologenes*, *Chrysebacterium gleum* and *Chryseobacterium hominis* are proven to cause infections in humans. They are commonly found in soil and water environments. They often colonize in water suppliers, taps, and sink basins; thereby forming reservoirs for infections in hospital environments.

Colonization of various medical devices containing fluids such as respirators, humidifiers, incubators for newborns, syringes, *etc.* have been well documented (*3-5*). Moreover, devices such as prosthetic valves and intravascular catheters have also been reported as a source of infection for this bacterium (*6*). In few other clinical settings, *Chryseobacterum* spp. have been described as causative agents of meningitis, pneumonia, endocarditis, bacteremia, infections of skin and soft tissue, and ocular infections (*7-13*). Primarily, it is an opportunistic pathogen that infects newborns and immunocompromised individuals of different age groups (*14*).

There are limited data published on the clinical significance and antimicrobial susceptibility of *Chryseobacterium* spp. worldwide including India (*15*). Breakpoints are not available for this newly emerging pathogen. Our study aims to find the clinical significance and antimicrobial susceptibility pattern of different *Chryseobacterium* spp. isolated from various clinical specimens.

2. Materials and Methods

This was a prospective study conducted in the Department of Microbiology of a tertiary care hospital in North India. The duration of the study was 3 years from January 2017 to December 2019. Ethical clearance was obtained from the institute ethics committee. In this study, we aimed to find the recent trends and clinical correlation of various *Chryseobacterium species* in different clinical specimens.

2.1. Specimen collection

Isolates from consecutive clinical samples (blood, CSF,

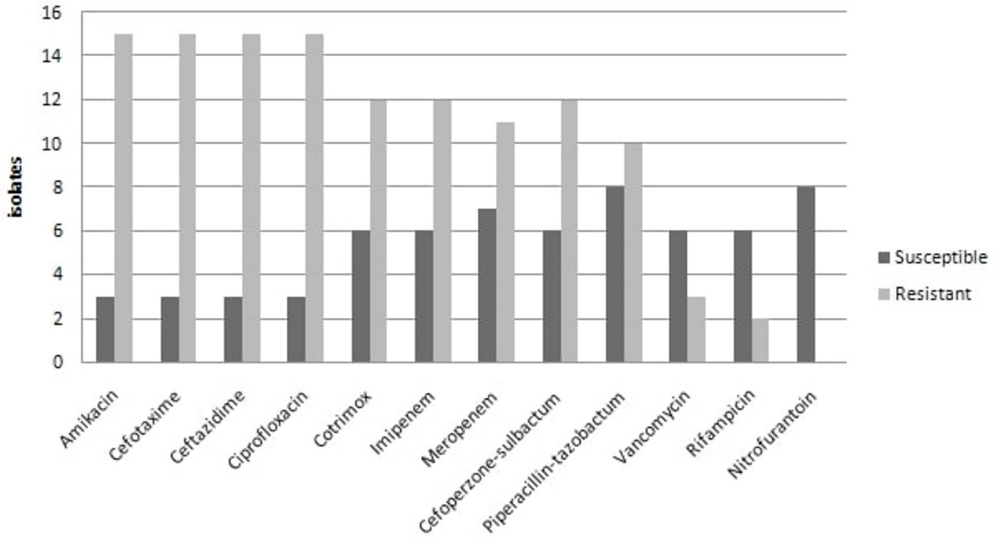


Figure 1. Antimicrobial susceptibility patterns of *C. Indologenes* isolates.

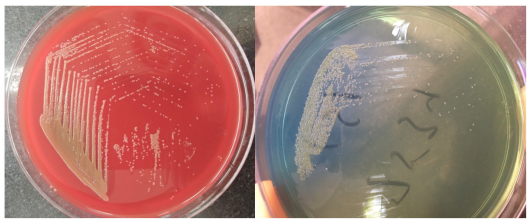


Figure 2. Dark-yellow colored colonies of *C. indologeneson* blood agar and CLED media.

urine, pus, respiratory samples, stool, pleural, peritoneal, pericardial fluids, bone marrow aspirates, catheter tips, *etc.*) had been processed for aerobic culture in the bacteriology laboratory during the defined period using standard operating procedures. Then final identification of the growth from culture was carried out using MALDI-TOF MS. Phenotypic characterization was also done using standard biochemical tests.

2.2. Drug susceptibility pattern

Antimicrobial susceptibility testing of 18 isolates of *C. indologenes* was performed on Muller Hinton agar (MHA) by Kirby Bauer disc diffusion method (Figure 1). SENTRY antimicrobial surveillance program was only the largest report published that showed data of 5 years (1997-2001) including antimicrobial susceptibility testing of *Chryseobacterium* spp. Because no standard guidelines for reporting Antimicrobial Susceptibility Testing (AST) for *Chryseobacterium* spp. were available, we performed AST using antimicrobial agents recommended by the SENRY antimicrobial surveillance program and the result was interpreted using break points for *Pseudomonas aeruginosa*, *Staphylococcus aureus* (for interpretation of cotrimoxazole, rifampicin, nitrofurantoin) and *Enterococcus* spp. (for interpretation of vancomycin) as recommended by the Clinical and Laboratory Standards Institute (CLSI) 2017 guidelines (*17*).

2.3. Clinical correlation

All the cases were prospectively followed to establish clinical correlation. Any changes in treatment/discharge/death were noted in the data sheet.

3. Results

A total of 20 isolates (*Chryseobacterium indologenes*, n = 18; and *Chryseobacterium gleum*, n = 2) were identified from various clinical specimens during the study period. Among the 18 isolates of *C. indologenes*, nine were isolated from blood, eight were from urine and one was from a BAL (bronchoalveolar lavage) specimen. Two *C. gleum* species were isolated from blood and BAL samples, respectively. Phenotypic identification of all isolates was also performed using conventional methods. The colonies on blood agar were observed to be yellowish in color, 1-2 mm, non-hemolytic, circular, low-convex and translucent with entire margins. No growth was observed on MacConkey agar. Urine samples were processed on CLED (Cysteine Lactose Electrolyte Deficient agar) media, which showed similar yellow colonies as on blood agar (Figure 2). The organism was found to be gram-negative non-motile bacilli with catalase positive and oxidase positive reactions. The Hugh and Leifson oxidative fermentative (OF) test showed an oxidative reaction. Indole was produced in tryptophan broth; whereas methyl red, urease production and citrate utilization tests were negative. *C. gleum* can be distinguished from *C. indologenes* by their ability to reduce nitrite to nitrogen. *Chryseobacterium* spp. can be easily differentiated phenotypically from other non-

Table 1. Clinical details of patients showing culture of *C. indologenes*

Specimen	No. of *Chryseobacterium* spp. (*n* = 18)	Medical Device (*n* = 10)	Comorbidities	Positive in Repeat isolation	Clinically significant
Urine	8 (44%)	3 (37.5%)	4 (50%)	3 (37.5%)	3
Blood	9 (50%)	6 (66.6%)	3 (33%)	1 (11%)	1
BAL	1 (5.5%)	1 (100%)	0	Nil	Nil

Medical device: Central line venous cather, Ventillator, urinary catheter. Comorbidities: Malignancy, immunosuppression.

fermenters as they are non-motile GNBs producing yellow colored colonies on blood agar, indole positive and inability to grow on MacConkey agar.

The patients, whose clinical specimen showed growth of *Chryseobacterium* spp. were prospectively followed up for their underlying conditions, co-morbidities, presence of indwelling devices, antibiotic history, change in therapeutic management (based on the culture and AST report), outcome, *etc.* More than 70% of the patients had at least one or more mechanical devices (*i.e.* urinary catheter, central venous catheter, or endotracheal tube) at the time of identification of infection by *Chryseobacterium* spp. (Table 1).

The susceptibility to rifampicin was observed as the highest (75%) followed by piperacillin-tazobactum (45%). Susceptibility against imipenem, meropenem, cotrimoxazole and cefoperazone-sulbactum were observed to be approximately 33%. Amikacin, cefotaxime and ceftazidime showed a least susceptibility pattern. All the 8 isolates of *C. indologenes* isolated from urine specimens were susceptible to nitrofurantoin. The AST of *C. gleum* showed maximum susceptibility to piperacillin-tazobactum and cefoperazone-sulbactum followed by cotrimoxazole, cefotaxime, imipenem and meropenem.

The isolation of *C. indologenes* was clinically significant among 4 out of 18 (22.2%) patients (three from urine samples and one from blood). The isolates from the urine samples were identified from acute myeloid leukemia (AML) patients and there was presence of the same growth on repeated occasions. Among the three patients, two of them had only fever and one of them had a urinary catheter in situ with urinary symptoms. Isolates from these three patients were susceptible to nitrofurantoin only. All of them were started on oral nitrofurantoin treatment after the susceptibility reports were generated. There was satisfactory clinical improvement with nitrofurantoin in all three patients and subsequent samples from these patients were sterile after completion of antibiotic treatment. A blood sample from central venous catheter in an elderly patient with acute kidney injury showed growth of *C. indologenes* on repeated occasions. On the basis of AST report, piperacillin-tazobactum was started and patient showed clinical improvement but unfortunately, he expired 4 days later due to multi-organ failure.

The two *C. gleum* strains were isolated from blood and BAL samples. Repeated blood cultures were found sterile. The isolate from blood was sensitive to all relevant antimicrobials except amikacin. An isolate from a BAL sample was sensitive to Cefoperazone-sulbactum and Piperacillin-tazobactum. The clinical significance of these isolates could not be established.

4. Discussion

Chryseobacterium spp. are emerging gram-negative bacilli belonging to the family of non-fermenters. With the use of MALDI-TOF MS, *Chryseobacterium* spp. are being increasingly identified. Since it exists in the hospital environment, a positive growth in culture has doubtful clinical significance. *Chryseobacterium* infections have been seen in patients with comorbidities like diabetes mellitus, chronic kidney disease, cardiovascular disease, chronic obstructive pulmonary disease and malignancies (*16*). Beside colonization in various medical devices by formation of bio-films other virulence factors like protease activity are yet to be explored and require further studies to establish their role in pathogenesis (*14,16*). There are limited data available determining the pathogenic role of *Chryseobacterium* spp. The SENTRY study (1997 to 2001) estimated the epidemiology and antimicrobial susceptibility pattern of *Chryseobacterium* infections worldwide, where the most common isolated species was *C. meningosepticum* followed by *C. indologenes* and *C. gleum* (*15*). The investigators described the differences in clinical features and anti-microbial susceptibility patterns between *C. indologenes* and *C. gleum* (*16*). They analyzed the database from 2005 and 2017 to identify patients with *Chryseobacterium* infections. A total of 84 isolates of *C. indologenes* and 42 isolates *C. gleum* were identified and studied during this period. Our study results were in agreement with this. There are no specific antimicrobial susceptibility testing guidelines available either from CLSI or EUCAST for the genus *Chryseobacterium*.

In our study, the most common species isolated was *C. indologenes* followed by *C. gleum*. *C. indologenes* was clinically correlated in 22% of the total patients who improved after changing the antibiotic regime. Urinary catheter remains an important risk factor for the causation of UTI by *C. indologenes* in most of the previously published literature. In the current study, two symptomatic patients without a urinary catheter showed significant growth of *C. indologenes*

on repeated occasions. Moreover, both the patients were immunosuppressed, which signifies there might be an increased chance of acquisition of infection in immunocompromised states. Because the numbers are very low in our study, follow up studies with adequate numbers may be helpful. In the current study, all urinary isolates observed were sensitive to nitrofurantoin, which is in agreement with a study conducted by Taneja *et al.* (*17*). More studies are needed to establish the efficacy of nitrofurantoin against this species.

A study by Kirby *et al.* showed more than 90% susceptibility of *C. indologenes* to piperacillin-tazobactum and cotrimoxazole (*15*). In our study, the susceptibility was 45% and 33% for piperacillin-tazobactum and cotrimoxazole, respectively. A recently published study by Lin *et al.* showed maximum susceptibility to minocycline (67.9%) followed by cotrimoxazole (47.9%).

Chryseobcterium gleum is also an uncommon pathogen rarely isolated from various clinical samples. There are limited data regarding *C. gleum*. Only a few case reports were reported from india. A study published by Rajendran *et al.* showed a case of nosocomial urinary tract infection by *C. gleum* in a diabetic elderly male patient with chronic renal disease and was successfully treated with ciprofloxacin (*18*). Few case reports published on *C. gleum* in patients suffering from chronic granulomatous diseases were possibly due to infection of the stent (*19,20*). In the current study, clinical correlation could not be established in both the *C. gleum* isolates and were considered to be contaminants. Regarding antimicrobial susceptibility patterns of *C. gleum*, our study showed maximum susceptibility to piperacillin-tazobactum, which coincides with the study done by Lin and his colleagues, published in 2019 (*16*).

In conclusion, there is increasing incidence of nosocomial infection due to multidrug resistant *Chryseobacterium* spp.. Early and accurate diagnosis of *Chryseobacterium* infection can provide an edge in appropriate management of the critically ill patients. With the help of advanced techniques like MALDI-TOF MS, these new emerging pathogens can be easily and accurately identified. There is a need to determine the breakpoints for various antibiotics against this bacterium across the world, to make a universal consensus for the antimicrobial susceptibility testing of *Chryseobacterium species*.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Bhuyar G, Jain S, Shah H, Mehta VK. Urinary tract infection by *Chryseobacterium indologenes*. Indian J Med Microbiol. 2012; 30:370-372.
2. Parte AC. LPSN – List of Prokaryotic names with Standing in Nomenclature (bacterio.net), 20 years on. Int J Syst Evol Microbiol. 2018; 68:1825-1829.
3. du Moulin GC. Airway colonization by Flavobacterium in an intensive care unit. J Clin Microbiol. 1979; 10:155-160.
4. Hoque SN, Graham J, Kaufmann ME, Tabaqchali S. Chryseobacterium (*Flavobacterium*) *meningosepticum* outbreak associated with colonization of water taps in a neonatal intensive care unit. J Hosp Infect. 2001; 47:188-192.
5. Lin YT, Jeng YY, Lin ML, Yu KW, Wang FD, Liu CY. Clinical and microbiological characteristics of *Chryseobacterium indologenes* bacteremia. J MicrobiolImmunol Infect. 2010; 43:498-505.
6. Chou DW, Wu SL, Lee CT, Tai FT, Yu WL. Clinical characteristics, antimicrobial susceptibilities, and outcomes of patients with *Chryseobacterium indologenes* bacteremia in an intensive care unit. Jpn J Infect Dis. 2011; 64:520-524.
7. Nulens E, Bussels B, Bols A, Gordts B, Van Landuyt HW. Recurrent bacteremia by *Chryseobacterium indologenes* in an oncology patient with a totally implanted intravascular device. Clin Microbiol Infect. 2001; 7:391-393.
8. Hendaus MA, Zahraldin K. *Chryseobacterium indologenes* meningitis in a healthy newborn: a case report. Oman Med J. 2013; 28:133-134.
9. Atıcı S, Ünkar ZA, Erdem K, Kadayifci EK, Karaaslan A, Memişoğlu AÇ, Soysal A, Toprak NÜ, Söyletir G, Özek E, Bakır M. Ventilator-associated pneumonia caused by *Chryseobacterium indologenes*: a rare infant case and review of the literature. Springerplus. 2016; 5:1741.
10. Bomb K, Arora A, Trehan N. Endocarditis due to *Chryseobacterium meningosepticum*. Indian J Med Microbiol. 2007; 25:161-162.
11. Bhagawati G, Bhardwaj A, Sajikumar R, Singh SP, Prajapati S. Bacteremia by *Chryseobacterium indologenes* in a patient with lung cancer: a clinical and microbiological investigation. Indian J Crit Care Med. 2019; 23:157-159.
12. Srinivasan G, Muthusamy S, Raveendran V, Joseph NM, Easow JM. Unforeseeable presentation of *Chryseobacterium indologenes* infection in a paediatric patient. BMC Res Notes. 2016; 9:212.
13. Bloom AH, Perry HD, Donnenfeld ED, Davis RG. *Chryseobacterium meningosepticum* keratitis. Am J Ophthalmol. 2003; 136:356-357.
14. Bloch KC, Nadarajah R, Jacobs R. *Chryseobacteriu mmeningosepticum*: an emerging pathogen among immunocompromised adults. Report of 6 cases and literature review. Medicine (Baltimore). 1997; 76:30-41.
15. Kirby JT, Sader HS, Walsh TR, Jones RN. Antimicrobial susceptibility and epidemiology of a worldwide collection of *Chryseobacterium* spp.: report from the SENTRY Antimicrobial Surveillance Program (1997-2001). J Clin Microbiol. 2004; 42:445-448.
16. Lin JN, Lai CH, Yang CH, Huang YH. Differences in clinical manifestations, antimicrobial susceptibility patterns, and mutations of fluoroquinolone target genes between *Chryseobacterium gleum* and *Chryseobacterium indologenes*. Antimicrob Agents Chemother. 2019; 63:e02256-18.
17. Kaur H, Mohan B, Hallur V, Raj A, Basude M, Mavuduru RS, Taneja N. Increased recognition of *Chryseobacterium* species as an emerging cause of nosocomial urinary tract infection following introduction of matrix-assisted

laser desorption/ionisation-time of flight for bacterial identification. Indian J Med Microbiol. 2017; 35:610-616.
18. Rajendran P, Muthusamy S, Balaji VK, Rakesh GJ, Easow JM. Urinary tract infection due to *Chryseobacterium gleum*, an uncommon pathogen. Indian J PatholMicrobiol. 2016; 59:551-553.
19. Rawat A, Vignesh P, Sharma A, Shandilya JK, Sharma M, Suri D, Gupta A, Gautam V, Ray P, Rudramurthy SM, Chakrabarti A, Imai K, Nonoyama S, Ohara O, Lau YL, Singh S. Infection profile in chronic granulomatous disease: a 23-year experience from a tertiary care center in North India. J Clin Immunol. 2017; 37:319-328.
20. Garg S, Appannanavar SB, Mohan B, Taneja N. Pyonephrosis due to *Chryseobacterium gleum*: a first case report. Indian J Med Microbiol. 2015; 33:311-313.

Received July 28, 2020; Revised October 26, 2020; Accepted December 11, 2020.

**Address correspondence to:*
Bimal Ku Das, Department of Microbiology, All India Institute of Medical Sciences, New Delhi-110029, India.
E-mail: tezpur.bimal@gmail.com

Released online in J-STAGE as advance publication January 12, 2021.

Brief Report

DOI: 10.5582/irdr.2020.03099

Cut-off value of C1-inhibitor function for the diagnosis of hereditary angioedema due to C1-inhibitor deficiency

Daisuke Honda[1], Isao Ohsawa[1,2,*], Satoshi Mano[1], Hisaki Rinno[1], Yasuhiko Tomino[1,3], Yusuke Suzuki[1]

[1] Department of Nephrology, Juntendo University Faculty of Medicine, Tokyo, Japan;
[2] Nephrology Unit, Internal Medicine, Saiyu Soka Hospital, Saitama, Japan;
[3] Medical Corporation SHOWAKAI, Tokyo, Japan.

SUMMARY Hereditary angioedema caused by C1-inhibitor (C1-INH) deficiency (HAE-C1-INH) is a rare autosomal dominant disease. Primary care physicians sometimes face difficulties in diagnosing HAE-C1-INH owing to fluctuations in C1-INH function levels influenced by blood sampling conditions. International major guidelines do not stipulate a cut-off value of C1-INH function for the diagnosis. We aimed to explore the distribution of C1-INH function levels in patients with HAE-C1-INH and elucidate the influence of blood sampling conditions using healthy volunteers' samples to confirm the cut-off value of C1-INH function. In 48 patients with HAE-C1-INH who visited the Juntendo University Hospital in Japan between 2013 and 2019, C1-INH function levels were evaluated for 160 samples during symptom-free periods and 147 samples during an acute attack. Fluctuations of C1-INH function level were also evaluated for 8 healthy volunteers, wherein the samples were divided into 3 groups according to different sampling conditions. C1-INH function levels in all patients with HAE-C1-INH were found to be < 50%. The average C1-INH function level in healthy volunteers measured soon after blood collection in an appropriate sampling condition was 77% (61-92%) with some having lower C1-INH function levels than the reference value. C1-INH function levels fluctuated unstably in inappropriate sampling conditions. In conclusion, we can confirm that a < 50% C1-INH function level can be used as the diagnostic cut-off value for HAE-C1-INH. Moreover, it is necessary to repeat measurements of C1-INH function level in appropriate blood sampling conditions to accurately diagnose HAE-C1-INH.

Keywords cut-off value, C1-inhibitor, guideline, hereditary angioedema, Japan

1. Introduction

Hereditary angioedema caused by C1-inhibitor (C1-INH) deficiency (HAE-C1-INH) is an autosomal dominant disease that produces excess bradykinin, inducing unpredictable and recurrent acute subcutaneous or submucosal angioedema (*1-4*). Although HAE-C1-INH is a rare disease, estimated to affect around 1 in 50,000 individuals with no reported bias among different ethnic groups, only approximately 400-500 patients, much less than the estimated prevalence, have been diagnosed in Japan. The responsible gene *SERPING1* has been detected, although the severity and frequency of the disease vary even in the same family members (*5*). HAE-C1-INH can be life-threatening when severe edema develops in the upper respiratory tracts, and patients might undergo unnecessary abdominal surgical procedures for severe abdominal pain resulting from gastrointestinal edema without appropriate treatment for HAE-C1-INH (*6-8*). Additionally, it has been reported that it takes an average of 13.8 years from the onset of the initial symptoms to be diagnosed with HAE-C1-INH due to the low awareness of the disease in Japan (*8*). To improve these conditions, an early diagnosis of HAE-C1-INH with clear criteria is important.

Concerning the diagnosis of HAE-C1-INH, the guideline of the World Allergy Organization/the European Academy of Allergy and Clinical Immunology (WAO/EAACI) recommends that all patients suspected to have HAE-C1-INH should be assessed for blood levels of C1-INH function, C1-INH protein, and C4, and the tests should be repeated to confirm the diagnosis of HAE-C1-INH, if any of the levels are abnormally low (*9*). The C1-INH protein level test is not covered by health insurance in Japan, and the measurement is not necessarily performed, because the C1-INH function

level test is sufficient for the diagnosis of HAE-C1-INH. Moreover, the guideline of the Japanese Association for Complement Research revised in 2014, recommends that low levels of C1-INH function and C4 are effective indicators, but the number of blood analyses of these markers needed for the diagnosis of HAE-C1-INH is not suggested (*10*). Both guidelines did not refer to the cut-off value of C1-INH function for diagnosis of HAE-C1-INH. Therefore, primary care physicians sometimes face difficulties in the diagnosis of HAE-C1-INH owing to confusingly low C1-INH function levels (*11,12*).

In the present study, we aimed to investigate the distribution of C1-INH function levels in patients with HAE-C1-INH to confirm the cut-off value of C1-INH function for an early diagnosis of HAE-C1-INH. Furthermore, because we sometimes encounter some patients with low C1-INH function levels, we also aimed to explore the influence of blood sampling conditions before measuring the C1-INH function level and fluctuations in the C1-INH function levels using healthy volunteers' samples.

2. Materials and Methods

2.1. Patients and blood samples

The study enrolled 48 patients (16 males, 32 females) with a confirmed diagnosis of HAE-C1-INH at the Juntendo University Hospital in Tokyo, Japan. The mean age of the patients was 41.1 years (range, 20-71 years) during their inclusion in this study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board of Juntendo University (No. 25-325). Written informed consent was obtained from the patients. We collected serum samples from the patients, as well as data on C1-INH function levels from 160 samples during symptom-free periods and 147 samples during an acute attack (total: 307 samples) between 2013 and 2019. Serum samples were appropriately measured soon after blood collection.

We planned to mimic the actual situations of blood sampling in the primary clinics. Serum samples were also obtained from 8 healthy volunteers (volunteers A-D: male, volunteers E-H: female) with no clinical symptoms of angioedema or other basic diseases. The samples were divided into the following 3 groups to measure C1-INH function levels: *i*) centrifuge the samples immediately after blood collection before the measurement, which should be ideally performed in clinical situations, *ii*) allow the samples to stand at room temperature for 6-24 h after the blood collection until centrifugation and the measurement, and *iii*) centrifuge the samples immediately after the blood collection and allow it to stand at room temperature for 6-24 h before the measurement. The *ii*) and *iii*) conditions were set as experimental controls.

2.2. Laboratory data

All serum samples were evaluated for C1-INH function levels by chromogenic assay in a commercial company (Special Reference Laboratories: SRL, Tokyo, Japan). The reagent for the C1-INH function level measurement was produced by a commercial company (Berichrom C1-inhibitor, Siemens, Munich, Germany). Thus, the testing procedures and methods were unified with their automated analysis methods. The reference value of C1-INH function was 70-130% set by the manufacturer; SRL (*13*).

3. Results and Discussion

All enrolled patients with HAE-C1-INH presented < 50% of C1-INH function levels (Table 1). Particularly, C1-INH function levels were ≤ 25% in 114 samples (71.3%) during symptom-free periods and 136 samples (92.5%) during an acute attack, respectively. Moreover, the enrolled patients had not received plasma-derived human C1-INH concentrate within 3 days before each blood collection, whose half-life period is reported to be 64 h (*14*).

The average C1-INH function level in 8 healthy volunteers immediately after the blood collection in an appropriate condition was 77.0% (61-92%).

For the samples left to stand at room temperature for 6-24 h after the blood collection and until the centrifugation and measurement, the C1-INH function levels were continuously decreased in 3 volunteers (B, D, G), continuously increased in 2 volunteers (C, F), and inconsistently changed in the others (Figure 1). The maximum rate of fluctuation in C1-INH function level was 22.8% in volunteer G.

For the samples immediately centrifuged after the blood collection and left to stand at room temperature for 6-24 h until the measurement, the C1-INH function levels continuously decreased in 2 volunteers (D, G), continuously increased in a volunteer (C), and inconsistently changed in the others (Figure 2). The maximum rate of fluctuation in the C1-INH function level was 31.7% in volunteer B.

Theoretically, C1-INH production declines by 50% in patients with HAE-C1-INH, because this is an autosomal dominant disease with the *SERPING1* gene mutation. However, C1-INH is continuously consumed by the kallikrein-kinin system, complement system, fibrinolysis system, contact system, and coagulation system. Indeed, the C1-INH function levels in all 307 of the HAE-C1-INH samples did not reach 50% (Table 1), although the C1-INH function levels fluctuated in many patients. We can therefore conclude that HAE-C1-INH is mostly ruled out, if the C1-INH function level is ≥ 50%, and that the cut-off value of C1-INH function for diagnosing HAE-C1-INH is considered likely to be 50%.

Table 1. The distribution of C1-INH function levels in patients with HAE-C1-INH

Pt no.	Number of patients presenting C1-INH function level									
	During symptom-free periods					During an acute attack				
	C1-INH function level (%)					C1-INH function level (%)				
	≤ 25	26-29	30-39	40-49	≥ 50	≤ 25	26-29	30-39	40-49	≥ 50
1	6	1	1			4				
2	6	2								
3	3		1			7				
4	3	2				6	1			
5	3					22			1	
6	5	3				2				
7	7					21	2			
8	5	1				5				
9	2					35				
10	1	1	1							
11	1									
12	1	3	2							
13			1							
14	7									
15	1									
16	1									
17				1						
18		1								
19			1							
20	2	1					1			
21	3									
22	5	1				5			1	
23	5					2				
24		1		4				1	1	
25										
26	5	1				1				
27	4					1		1		
28	3	1				10	1		1	
29			1							
30	4	1								
31	7									
32	1	4	1							
33	4									
34	2					9				
35	1	3	1							
36	1									
37	1									
38	2									
39	1		1			5				
40	1									
41	1									
42	3									
43	4									
44	1					1				
45		1								
46	1									
47		1								
48		1								
	114	30	11	5	0	136	5	2	4	0
Total	160					147				
	307									

All enrolled patients with HAE-C1-INH presented < 50% of C1-INH function levels. C1-INH function levels were ≤ 25% in 114 samples (71.3%) during symptom-free periods and 136 samples (92.5%) during an acute attack, respectively. Moreover, the enrolled patients had not received plasma-derived human C1-INH concentrate within 3 days before each blood collection, whose half-life period is reported to be 64 h. C1-INH, C1-inhibitor; HAE, Hereditary angioedema; Pt no., Patient number.

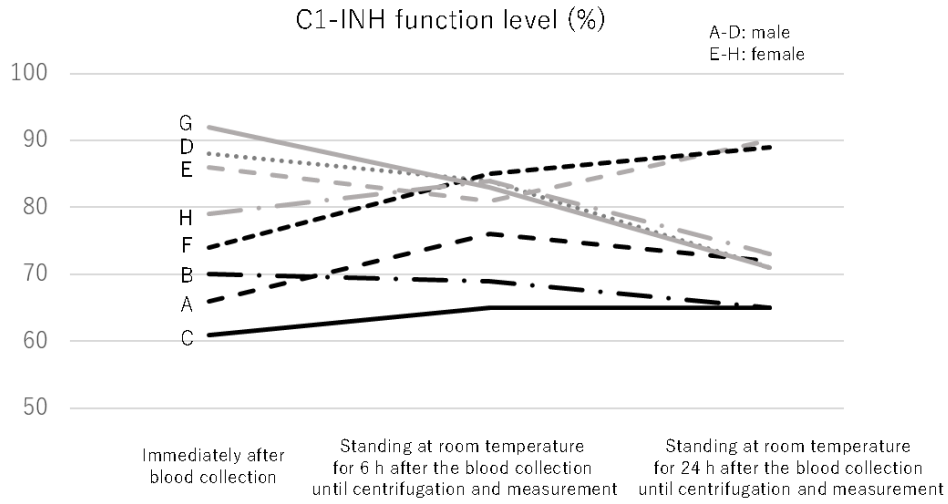


Figure 1. C1-inhibitor (C1-INH) function levels of the samples from 8 healthy volunteers standing at room temperature for 6-24 h after blood collection until centrifugation and measurement. The figure shows the fluctuations of C1-INH function levels in 8 healthy volunteers. The samples were left to stand at room temperature for 6-24 h after blood collection until centrifugation and measurement. C1-INH function levels were continuously decreased in volunteers B, D, and G, continuously increased in volunteers C and F, and inconsistently changed in the others. The maximum rate of fluctuation in C1-INH function level was 22.8% in volunteer G.

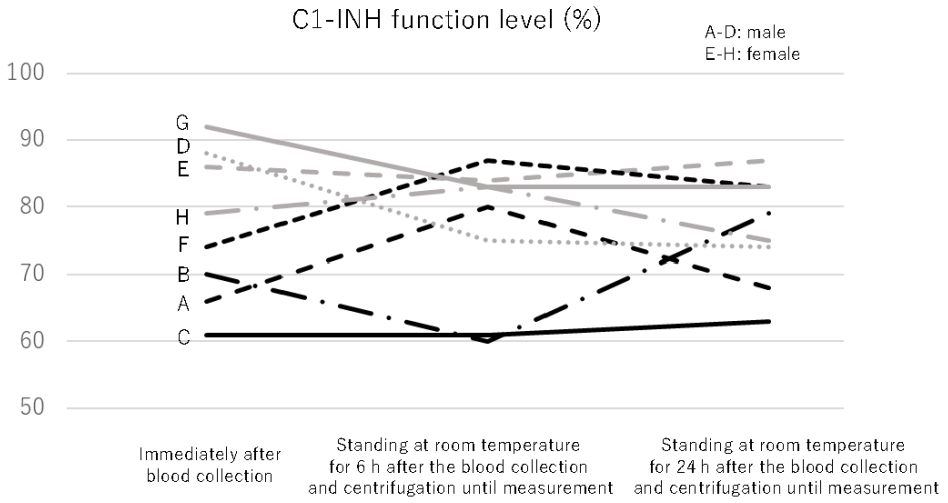


Figure 2. C1-inhibitor (C1-INH) function levels of samples from 8 healthy volunteers standing at room temperature for 6-24 h after blood collection and centrifugation until measurement. The figure shows fluctuations of C1-INH function levels in 8 healthy volunteers. The samples were centrifuged immediately after blood collection and left to stand at room temperature for 6-24 h before measurement. C1-INH function levels were continuously decreased in volunteers D and G, continuously increased in volunteer C, and inconsistently changed in the others. The maximum rate of fluctuation in C1-INH function level was 31.7% in volunteer B.

The reference value of C1-INH function is set as a wide range of 70-130%, because C1-INH function levels can fluctuate due to some biological factors. C1-INH is reported to decline during late phase of pregnancy and it also decreases due to liver disease, malignant disease, extracorporeal circulation therapy, disseminated intravascular coagulation, multiple organ failure, arteriosclerosis obliterans, and infection (*15*). Moreover, in patients with low levels of C1-INH function, the influence of blood sampling conditions before measurement should be taken into consideration. We have previously experienced some cases of non-HAE-C1-INH patients with angioedema, showing low C1-INH function levels at the first measurement, but not at the second measurement as presented below.

Case 1, 15-year old, female: The patient experienced recurrent swelling around the mouth at the age of 15 years. After blood analysis, her family doctor suspected HAE-C1-INH because of the C1-INH function level being < 25%. Although she had a history of angioedema in her paternal family, her blood analysis result in our hospital showed normal C1-INH function levels (96%).

Because she originally had urticaria and pollinosis with a high serum level of IgE (269 IU/mL), she was diagnosed with histamine-mediated angioedema (*16*). We found that the result of blood analysis for C1-INH function level performed in the previous hospital had been obtained using stocked serum 7 days after blood collection, which might have led to the incorrect C1-INH function level.

In this study, the fluctuations of C1-INH function level were observed at about 20-30% according to the inappropriate conditions as shown in Figures 1 and 2. Furthermore, the results of measuring the samples would be naturally impaired by poor testing conditions as we experienced in case 1.

Moreover, we experienced another case of a non-HAE-C1-INH patient with angioedema as presented below.

Case 2, 32-year old, female: After beginning to take estrogen pills at the age of 30 years, the patient experienced facial swelling, for which she visited her home doctor. The result of her blood analysis indicated HAE-C1-INH owing to the presence of low C1-INH function levels (63%). She had no family history of angioedema, and the result of her blood analysis in our hospital showed normal levels (C1-INH function level of 93.8%). We were unable to identify the primary problem associated with the blood collection and measurement procedure in the previous hospital; therefore, we considered that the estrogen pills may have caused drug-induced angioedema (*17*).

Thus, the measurement results of C1-INH function levels might originally show wide variations that cannot be explained simply by blood sampling conditions before the measurement. First, in this study, the obvious difference or tendency was not observed between different blood sampling conditions before measurement as shown in Figure 1 and Figure 2. Second, considering the accuracy and precision of the reagent for C1-INH function levels measurement used in the present study, because Siemens reveals that its accuracy is < 15%, and its coefficient of variation is < 10%, we can say that the reagent used for C1-INH function levels is reliable (*18*). Third, because the measurement of C1-INH function levels was usually outsourced in most of the Japanese hospitals, the testing procedures and methods were unified according to their automated analysis methods. Thus, if blood sampling conditions have an influence on the results, the C1-INH function levels observed in this study should show consistent changes and tendencies over time. Therefore, we need to recognize that the measurement of C1-INH function levels might be unexpectedly unstable.

Additionally, in this study, 2 healthy volunteers (A, C) presented lower C1-INH function levels than the reference value. In particular, volunteer C who showed the lowest C1-INH function level immediately after blood collection, consistently showed 61-65% in all conditions as shown in Figure 1 and Figure 2. Because we were assured that volunteer C did not have any disease including angioedema, this result means that some healthy people could present lower C1-INH function levels than the reference value.

In conclusion, we can confirm that < 50% of C1-INH function level can be used as the diagnostic cut-off value for HAE-C1-INH. Moreover, it is necessary to repeat measurements of C1-INH function level in an appropriate blood sampling condition to accurately diagnose HAE-C1-INH as recommended by the guideline of WAO/EAACI. We hope that more samples will be included in the future to provide more sufficient data as evidence to support this conclusion.

Funding: This study was partly supported by the MEXT (Ministry of Education, Culture, Sports, Science and Technology) and JSPS (Japan Society for the Promotion of Science) KAKENHI; grant number 19K17917.

Conflict of Interest: D.H has received honoraria as a speaker from Takeda Pharmaceutical Company. I.O has received honoraria as a speaker/advisor from BioCryst, CSL Behring and Takeda Pharmaceutical Company. S.M, H.R, Y.T, and Y.S have no financial conflicts of interest to declare.

References

1. Zuraw BL, Christiansen SC. HAE pathophysiology and underlying mechanisms. Clin Rev Allergy Immunol. 2016; 51:216-229.
2. Zuraw BL. Clinical practice. Hereditary angioedema. N Engl J Med. 2008; 359:1027-1036.
3. Bork K, Meng G, Staubach P, Hardt J. Hereditary angioedema: new findings concerning symptoms, affected organs, and course. Am J Med. 2006; 119:267-274.
4. Longhurst H, Cicardi M. Hereditary angio-oedema. Lancet. 2012; 379:474-481.
5. Fukunaga A, Tsuchiyama S, Lee K, Washio K, Hashimura C, Horiuchi T, Nishigori C. The relationship between complement levels and disease activity in Japanese family cases of hereditary angioedema with C1-INH deficiency. Allergol Int. 2018; 67:518-520.
6. Bork K, Hardt J, Witzke G. Fatal laryngeal attacks and mortality in hereditary angioedema due to C1-INH deficiency. J Allergy Clin Immunol. 2012; 130:692-697.
7. Honda D, Ohsawa I, Shimizu Y, Maiguma M, Hidaka T, Suzuki H, Io H, Mano S, Takahara H, Rinno H, Tomino Y, Suzuki Y. Suffocation due to acute airway edema in a patient with hereditary angioedema highlighted the need for urgent improvements in treatment availability in Japan. Intern Med. 2018; 57:3193-3197.
8. Ohsawa I, Honda D, Nagamachi S, Hisada A, Shimamoto M, Inoshita H, Mano S, Tomino Y. Clinical manifestations, diagnosis, and treatment of hereditary angioedema: survey data from 94 physicians in Japan. Ann Allergy Asthma Immunol. 2015; 114:492-498.
9. Maurer M, Magerl M, Ansotegui I, *et al.* The international WAO/EAACI guideline for the management of hereditary angioedema – the 2017 revision and update. Allergy.

2018; 73:1575-1596.
10. Horiuchi T, Ohsawa I, Okada, H, *et al*. Hereditary Angioedema (HAE) Guidelines - Revised 2014 Edition. Created by the Japanese Association for Complement Research *http://square.umin.ac.jp/compl/common/images/disease-information/hae/HAEGuideline2014.pdf* (accessed October 7, 2010). (in Japanese)
11. Ohsawa I, Honda D, Nagamachi S, Hisada A, Shimamoto M, Inoshita H, Mano S, Tomino Y. Clinical and laboratory characteristics that differentiate hereditary angioedema in 72 patients with angioedema. Allergol Int. 2014; 63:595-602.
12. Honda D, Ohsawa I, Sato N, Inoshita H, Mano S, Tomino Y, Suzuki Y. Diminished capacity of opsonization and immune complex solubilization, and detection of anti-C1q antibodies in sera from patients with hereditary angioedema. Allergol Int. 2017; 66:603-609.
13. Serum immunological test, C1 esterase inhibitor activity, SRL, Japan. *https://test-guide.srl.info/hachioji/test/detail/011656409* (accessed October 7, 2010). (in Japanese)
14. Brackertz D, Isler E, Kueppers F. Half-life of Cl-INH in hereditary angioneurotic oedema (HAE). Clin Allergy. 1975; 1:89-94.
15. Caliezi C, Wuillemin WA, Zeerleder S, Redondo M, Eisele B, Hack CE. C1-Esterase inhibitor: an anti-inflammatory agent and its potential use in the treatment of diseases other than hereditary angioedema. Pharmacol Rev. 2000; 52:91-112.
16. Ohsawa I, Honda D, Hisada A, Inoshita H, Onda-Tsueshita K, Mano S, Sato N, Nakamura Y, Shimizu T, Gotoh H, Goto Y, Suzuki Y, Tomino Y. Clinical features of hereditary and mast cell-mediated angioedema focusing on the differential diagnosis in Japanese patients. Intern Med. 2018; 57:319-324.
17. Bork K, Wulff K, Witzke G, Stanger C, Lohse P, Hardt J. Antihistamine-resistant angioedema in women with negative family history: estrogens and F12 gene mutations. Am J Med. 2013; 126:1142.e9-14.
18. Package insert, berichrom C1 inhibitor, SYSMEX. *https://sysmex-support.com/jp/section/coagulation/package_insert* (accessed October 7, 2010). (in Japanese)

Received August 30, 2020; Revised October 7, 2020; Accepted November 18, 2020.

**Address correspondence to:*
Isao Ohsawa, Nephrology Unit, Internal Medicine, Saiyu Soka Hospital, 1-7-22 Matsubara, Soka City, Saitama 340-0041, Japan.
E-mail: i.osawa@saiyukai.com

Released online in J-STAGE as advance publication December 2, 2020.

Case Report

DOI: 10.5582/irdr.2020.03146

Tetraploid acute promyelocytic leukemia with double translocation t (15,17) PML/RARA: the first case report in Croatia and Europe

Vlatka Periša[1,2,*], Dorian Laslo[1], Ivana Franić-Šimić[3], Jasminka Sinčić-Petričević[2]

[1] Faculty of Medicine Osijek, University Josip Juraj Strossmayer of Osijek, Osijek, Croatia;
[2] University Hospital Centre Osijek, Clinic of Internal Medicine, Department of Hematology, Osijek, Croatia;
[3] Clinical Hospital Centre Zagreb, Clinical department for laboratory diagnostics, Zagreb, Croatia.

SUMMARY Acute promyelocytic leukemia (APL) is characterized by the translocation t (15;17)(q22;q21) cytogenetic abnormality in the majority of cases. In most of the cases the cells of APL have normal, diploid karyotype. There are very few cases presented with very rare tetraploid karyotype with double translocation t(15;17)(q22;q12). We report the first case of tetraploid APL with double translocation t(15, 17) in Europe. A 66-year old male patient presented with dyspnea and unexplained dental bleeding. Blood work showed a white blood cell count of 1x109/L, hemoglobin was 124 g/L, platelet count was 61x109/L and fibrinogen level was low (1.4 g/L). Cytogenetics showed a tetraploid karyotype. Fluorescence in situ hybridization analysis proved existence of clonal cells with translocation t (15,17) in 15% of metaphase nuclei and tetraploid subclonal cells with the same translocation in 70% of metaphase nuclei. Findings were consistent with APL, tetraploid variant and the patient started all-trans retinoic acid (ATRA) treatment. The patient achieved complete remission in 2 months and completed three consolidation therapy cycles with ATRA, idarubicin or mitraxontrate. Currently, the patient is undergoing maintenance therapy with ATRA, 6-mercaptopurine and weekly methotrexate.

Keywords acute promyelocytic leukemia, all trans-retinoic acid (ATRA), PML-RARA, tetraploid

1. Introduction

Acute promyelocytic leukemia (APL) is a type of acute myeloid leukemia (AML), classified according to the French-American-British (FAB) classification as AML-M3 and accounts for about 10-15% of all AMLs. APL is characterized by a balanced reciprocal translocation (t) between chromosomes 15 and 17 resulting in the fusion of the retinoic acid receptor alpha (RARA) and promyelocytic leukemia (PML) genes in the majority of cases. In most of the cases the cells of APL have normal, diploid karyotype (*1,2*). There are very few cases presented with very rare tetraploid karyotype with double translocation t (15;17)(q22;q12). Tetraploidy has only been reported in 16 cases of APL in the literature, with cases reported in the Far East countries, Australia, United States of America, Malaysia and Greece (*3-5*).

We searched PubMed using key words "acute promyelocytic leukemia" and "tetraploid" and found 20 results. None of those results was related to Croatia, thus, to our knowledge, our case is the first reported tetraploid APL with double t (15;17)/PML-RARA in an adult from Croatia. Furthermore, until now only one APL with tetraploidy case report has been published in Europe (5) but in that case report t (15;17)(q22;q12) has not been proven. Accordingly our case report is the first case of tetraploid APL with double t (15;17)(q22;q21).

2. Case Report

We present a case of a 66-year old male patient who presented with dyspnea, and dental bleeding. Blood work showed a white blood count of 1×10^9/L with 39% neutrophils, 49% lymphocytes and 5% monocytes. The hemoglobin was 124 g/L and the platelet count was 61×10^9/L.

The prothrombin and activated partial thromboplastin time were normal but fibrinogen level was low (1.4 g/L). The bone marrow showed numerous large promyelocytes (54%) that contained irregular bilobed nuclei, abundant cytoplasm with granularity and few Auer Rods. Flow cytometry showed a population of large immature cells phenotypically positive for cluster of differentiation (CD)13, CD33, myeloperoxidase (MPO), CD117, CD56, CD64, CD2 and negative

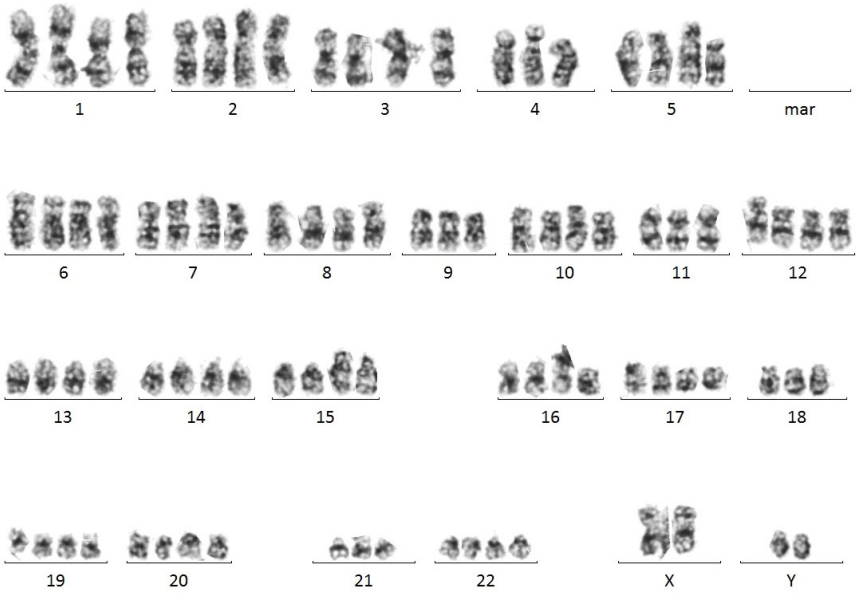


Figure 1. Karyogram of tetraploid subclone of acute promyelocytic leukemia cells.

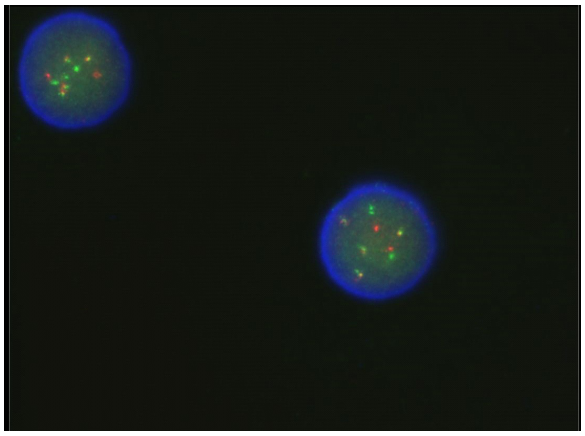


Figure 2. Fluorescence in situ hybridization analysis of tetraploid translocation t (15,17) (q21, q22) with PML/RARA probe.

for Human Leukocyte Antigen – DR isotype (HLA-DR) and CD11c which referred to promyelocytes. Cytogenetics showed a tetraploid karyotype as follows: 46,XY,t(15;17)(q22;q21)/92,XXYY,t(15;17)(q22;q21) × 2 (Figure 1). Fluorescence in situ hybridization (FISH) analysis proved existence of clonal cells with translocation t (15, 17) in 15% of metaphase nuclei and tetraploid subclonal cells with the same translocation t (15,17) in 70% of metaphase nuclei (Figure 2). PML/RARA copies were identified by a reverse transcriptase-polymerase chain reaction (RTPCR).

Findings were consistent with APL, tetraploid variant. Induction therapy with all trans-retinoic acid (ATRA) and idarubicin were completed with no complications. Post induction bone marrow cytogenetics revealed a normal male karyotype and FISH and PCR studies showed no PML/RARA fusion products, consistent with APL in molecular remission. The patient achieved a complete remission in 2 months and completed three consolidation therapy cycles with ATRA, idarubicin or mitraxontrate with 28 months of follow up. Repeat bone marrow examination and molecular analysis after completion of consolidation treatment showed the patient remained in morphological and molecular remission. The latest molecular analysis which was done 50 weeks after consolidation therapy revealed undetectable PML-RARA fusion copies. Currently, the patient is undergoing maintenance therapy with ATRA, 6-mercaptopurine and weekly methotrexate.

3. Discussion

In most cases APL is characterized with giant and bizarre blast cells translocation t (15,17) (q21,q22) and synthesis of fusion transcriptional product PML/RARA. Initial treatment of APL includes application of ATRA with or without additional chemotherapeutics or idarubicine. Tetraploidy with double translocation t (15,17) is an extremy rare finding. To our knowledge this is the 17th published case of tetraploid APL (*3,4*).

Clinical presentation of APL (with or without tetraploidy) is the same. It is characterized by sudden start of heavy hemorrhage associated with high risk of disseminated intravascular coagulopathy development (*6*). It seems that tetraploidy does not influence the inital treatment response in patients with tetraploidy with double translocation (*7*). Counting this case report 14 out of 17 patients had complete cytogenetic and hematological remission as a result of initial treatment using ATRA with or without additional chemotherapeutics. It seems that there is no positive correlation between age and long term outcome.

Table 1. The most important clinical findings of previously published tetraploid APL case reports*

No.	Country	Authors	Age/Sex	Immunophenotype	Cytogenetics
1	Japan	Kaito *et al.*	56/M	CD2+, CD13+, CD33+, CD34+, CD56+, HLA-DR-	92,XXYY,t(15;17)(q22;q21) × 2
2	South Korea	Oh *et al.*	50/F	CD2+, CD13+, CD33+, CD34-, CD56-, HLA-DR-	92,XXXX,t(15;17)(q22;q21) × 2
3	Japan	Morita *et al.*	50/M	CD2+, CD13+, CD33+, CD34+, HLA-DR+	45,XY,add(1)(p36),9, der(15)t(15;17),17,add(20)(q13),21,mar1,mar2[2]/ 46,idem,mar3[6]/45,idem, del(11)(p11),add(13)(p11), 18,21,mar1,mar2[2]/86,XX,Y,Y,add(6)(p21) × 2,8,9,11,12,der(15)t(15;17)(q22;q11~q21) × 2, 16,17,17,18,19,mar4,mar5[2]/46,XY[5]
4	Australia	Mohamed *et al.*	32/M	CD13+, CD33+, CD34-, CD117+, HLA-DR-	92,XXXX,t(15;17)(q22;q21) × 2
5	United States	Ravella *et al.*	48/M	CD2-, CD13+, CD33+, CD34-, CD56-, CD117-, HLA-DR-	92,XXYY,t(15;17)(q22;q21)X2[4]/92,XXYY,add(5)(q22),t(15;17) (q22;q21)X2[3]/46,XY[13]
6	China	Pan *et al.*	21/M	CD2+, CD13+, CD33+, CD117+	46,XY,t(15;17)[18]/92,XXYY,t(15;17)× 2[6]/46,XY
7	China	Pan *et al.*	26/M	CD2+, CD13+, CD33+	92,XXYY,t(15;17) × 2
8	China	Pan *et al.*	68/M	CD2+, CD13+, CD33+, MPO+	92,XXYY,t(15;17) × 2[5]/46,XY
9	China	Pan *et al.*	40/M	CD13+, CD33+	92,XXYY,t(15;17) × 2
10	China	Pan *et al.*	38/M	CD33+	92,XXYY,t(15;17) × 2[18]/46,XY
11	China	Au *et al.*	24/M	CD2+, CD13+, CD33+, MPO+	73~89,XXY,-Y[18],-3[10],-5[9],-7[4],-9[7],-11[9],-14[10],-15[9],t(15;17)[10],t(15;17)[4],der(15)t(15;17)[4],-17[8],-18[7],-19[9],-20[18],+mar1[9],+mar2 × 2[10],+mar3[7][cp10]/46,XY
12	Japan	Kojima *et al.*	53/M	Not available	92,XXYY,del(2)(q?),t(15;17)(q22;21)x2[4]/46,XY[16]
13	Japan	Kuyama *et al.*	56/M	CD2-, CD13+, CD33+, CD34-, CD56-, HLA-DR	90[1/6]/91[1/6]/92,XXYY,t(15;17)(q22;q12) × 2,-16,-16, +2mar[4/6]
14	Greece	Matsouka *et al.*§	49/M	CD13+, CD33+, CD34+, CD38+, CD56+, HLA-DR-	92,XXYY
15	United States	Dalia *et al.*	51/M	CD7-, CD13+, CD33+, CD34+, CD56+, CD117+, MPO+, HLA-DR-	91,XXYY,-9,(15;17)(q24;q21.1) × 2
16	Malaysia	Tay Za *et al.*	57/M	CD13+, CD33+, CD117+, cMPO+, CD34+(heterogenous), CD64+, CD56+,CD2+, HLA-DR-, CD11b-, CD15-, CD14-, CD41, CD61-, CD71-, glycophorin-	89~92,XXYY,t(15;17) (q22;q21) × 2[7]/46,XY[5]
17	Croatia	Our case	66/M	CD13+, CD33+, MPO+, CD117+, CD 56+, CD64+, CD 2+ HLA-DR-, CD11c-	46,XY,t(15;17)(q22;q21)/92,XXYY,t(15;17)(q22;q21) × 2

*Data from published studies (Ref. *3-5*). §Tetraploidy without double t (15;17)

Three patients at ages under 60 years died despite ATRA treatment, and on the other hand three patients above the ages of 50 and one patient at the age of 68 achieved complete remission (*4*). Influence of additional chromosome anomalies (ACA) in long-term survival of the patients with non-tetraploid APL was analyzed and it was estimated that trisomy 8 is the most frequent ACA, but it was concluded that ACAs have no influence on treatment response or survival (*8*).

Counting this case report, until now only 17 case reports were published and 11 of them were patients from the Far East (Japan, China, Korea). Table 1 summarizes the 17 cases of tetraploidy APL (*3-5*). Since there was no difference in therapy response or long-term survival between patients from Far East and other countries, that difference in patients distribution could be caused by publicational bias (*4,5*) especially if we take into account that only two case repotrs were published from Western hemisphere countries (*4,9*), and counting this case report, only two from Europe (*5*). What could be significant is that male sex could have greater risk for tetraploid APL development, since only 1 out of 17 patients was a woman at the age of 50 (*6*). Complete remission was achieved in 14 out of 17 cases, but long-term survival of the patients with tetraploid APL compared to those with non-tetraploid APL seems to be lower, but for now the number of tetraploid APL patients is too low for any firm conclusions about long-term survival.

4. Conclusion

We report the first case of tetraploid APL with double translocation t (15,17) in Europe. Based on the previous reports, APL with tetraploid karyotype appears to have a similar clinical outcome to diploid APL. Nevertheless, it is difficult to draw a firm conclusion in regards to the long-term prognosis of APL patients with a tetrapolid karyotype due to the very small number of such cases reported. We encourage others to report cases of tetraploidy APL in order to understand this rare cytogenetic subgroup better.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Jimenez JJ, Chale RS, Abad AC, Schally AV. Acute promyelocytic leukemia (APL): a review of the literature. Oncotarget. 2020; 11:992-1003.
2. Cicconi C, Lo-Coco F. Current management of newly diagnosed acute promyelocytic leukemia. Ann Oncol. 2016; 27:1474-1481.
3. Tay Za K, Jackson N, Chin EFM. Tetraploid/near-tetraploid acute promyelocytic leukaemia with double (15;17) translocation. Malaysian J Pathol. 2020; 42:127-130.
4. Dalia SM, Horna P, Zhang L. Tetraploidy acute promyelocytic leuemia with double t(15;17)/PML-RARA, a case report with review of literature. Int J Clin Exp Pathol. 2014; 7:5363-5368.
5. Matsouka P, Sambani C, Giannakoulas N, Symeonidis A, Zoumbos N. Polyploidy in acute promyelocytic leukemia without the 15:17 translocation. Haematologica. 2001; 86:1312-1313.
6. Oh SH, Park TS, Kim HH, Chang CL, Lee EY, Son HC, Chung JS, Cho GJ. Tetraploid acute promyelocytic leukemia with double t(15;17) and PML/RARA rearrangements detected by fluorescence in situ hybridization analysis. Cancer Genet Cytogenet. 2003; 145:49-53.
7. Pan J, Xue Y, Qiu H, Wu Y, Wang Y, Zhang J, Shen J. Tetraploid clone characterized by two t(15;17) in five cases of acute promyelocytic leukemia. Cancer Genet Cytogenet. 2009; 188:57-59.
8. Ono T, Takeshita A, Iwanaga M, Asou N, Naoe T, Ohno R; Japan Adult Leukemia Study Group. Impact of additional chromosomal abnormalities in patients with acute promyelocytic leukemia: 10-year results of the Japan Adult Leukemia Study Group APL97 study. Haematologica. 2011; 96:174-176.
9. Ravella PM, Liu D, Kojima K, Weisberger J, Miura O, Kuyama J, Au W, Seiter K. Acute promyelocytic leukemia with tetraploid karyotype: first report in the Western hemisphere and update of previous reports. Leuk Res. 2011; 35:93-95.

Received November 15, 2020; Revised January 20, 2021; Accepted January 27, 2021.

**Address correspondence to:*
Vlatka Periša, Faculty of Medicine Osijek, University Josip Juraj Strossmayer of Osijek, Osijek, Croatia and University Hospital Centre Osijek, Clinic of Internal Medicine, Department of Hematology, Osijek, Croatia.
E-mail: vlatkaperisa@gmail.com

Released online in J-STAGE as advance publication February 5, 2021.

Letter

DOI: 10.5582/irdr.2020.03116

A rare challenge in general surgery: double surgical procedure for large and small bowel obstruction in a patient with Gerstmann-Sträussler-Scheinker syndrome

Andrea Costanzi[1], Michela Monteleone[1,*], Valter Berardi[2], Angelo Miranda[2], Giulio Mari[2], Dario Maggioni[2]

[1] General Surgery Unit, San Leopoldo Mandic Hospital, Merate, ASST Lecco, Italy;
[2] General and Emergency Surgery Unit, Desio Hospital, ASST Monza, Italy.

SUMMARY Gerstmann-Sträussler-Scheinker syndrome (GSS) is a rare, infectious syndrome related to a mutation in the prion protein gene. Described here are the challenges posed by surgery for a patient with GSS. A 61-yr-old woman with GSS was admitted to this department and underwent surgery twice for large and small bowel obstruction. This is the first report of two major surgical procedures in a patient with GSS. Experiences with this case and precautions when using a disposable device during endotracheal intubation and a surgical procedure to manage a patient with GSS are described.

Keywords Gerstmann-Sträussler-Scheinker syndrome, general surgery, bowel obstruction

Gerstmann-Sträussler-Scheinker Syndrome (GSS) is a rare prion disease (PD). This familial (autosomal dominant), fatal neurodegenerative disease affects patients from 20 to 60 years of age and is related to a prion protein (PrP) mutation in the group of subacute spongiform encephalopathies (SEs) (*1*). Human SEs include Creutzfeldt-Jakob disease, GSS, Kuru, and fatal familial insomnia. GSS is characterized by amyloid deposition in the cerebral parenchyma or blood vessels (*2*). Symptoms include dysarthria, progressive cerebellar truncal ataxia, pyramidal signs, and adult-onset dementia. Some studies have suggested that a PD might be transmitted by blood or plasma-derived products from patients during the prodromal stage. In animal studies, intracerebral inoculation of infected cells has been associated with development of disease, and infectivity was also detected in the blood (*3*). Given the lack of information, the resistance of PrP to conventional sterilizing measures is a major problem. Current recommendations are to identify at-risk patients and to use disposable devices during endotracheal intubation, spinal anesthesia, and surgery (*4*).

A 61-yr-old woman was admitted to this Hospital for abdominal pain. At the age of 53, she complained of dysarthria, spastic paraparesis, cognitive decline, amyotrophy, depression, cerebellar ataxia, pseudo-bulbar palsy, and absent tendon reflexes in the lower limbs. Her father and sister had the same symptoms. A typical mutation of the 102nd amino acid (PRNP-p.D202N) and a drastic change were found in the normal prion gene. She was then diagnosed with classical GSS. Her Unified Parkinson's Disease Rating Scale III (UPDRSIII) score was 26 and her Hoehn and Yahr scale score was 3 (*5*). The patient was admitted to the emergency department for abdominal pain. Laboratory results revealed an increased white blood cell count (WBC) [14×10^9/L (NV 4–10×10^9] and elevated C-reactive protein (CRP) level [33.3 mg/L (NV < 1.0)]. A CT scan of the abdomen and thorax revealed sigmoid volvulus. The endoscopic evaluation confirmed sigmoid stenosis and mucosal necrosis. After an interview with the patient's caregivers who are actively present in the patient's life and fully dedicated to her medical and physical care and a consultation with anesthesiologists and physiotherapists, a plan was formulated to use general anesthesia with tracheal intubation in order to perform an explorative laparotomy.

A volvulus of the dolichosigmoid colon with bowel obstruction and ischemia was detected intraoperatively. In order to avoid anastomotic complications, the patient underwent a Hartmann's resection with end-colostomy. The highest level of protection was used during surgery. In addition to disposable masks and caps, all medical staff wore gowns and gloves. Disposable surgical instruments and anesthetic equipment were used. The scrub team was equipped with full personal protective equipment. The number of surgical, nursing, and anesthesia team members was limited to

Table 1. Patient characteristics

	Age (yrs)	Neurological Symptoms	Abdominal Symptoms	WBC ($\times 10^9$/L)	CRP (mg/L)	Radiological Findings	Surgical Procedure	Anesthetic Outcome	Surgical Outcome
1st surgery	61	Dysarthria; Spastic Paraparesis; Dementia; Cerebellar Ataxia; Pseudo-bulbar Palsy; Tendon Reflexes Absent	Abdominal Pain; Nausea and Vomiting; Bowel Obstruction	14	33	Colonic Distension; Bowel Obstruction; Sigmoid Volvulus; No Signs of Perforation	Laparoscopic Hartmann's Resection with end Colostomy	No Airway Obstruction; Extubated on Day 1 Postop Percutaneous mini-tracheostomy	Uneventful; Discharged on Day 6 Postop
2nd surgery	62	Stable	Abdominal Pain; Nausea and Vomiting; Small Bowel Obstruction	12	9	Bowel Distention; Closed-loop Obstruction	Laparotomic Small Bowel Resection; PEG Tube Placement	Uneventful	Uneventful; Discharged on Day 9 Postop

yrs: years; WBC: white blood cell count; CRP: C-reactive protein.

the minimum required to perform the surgery. After confirming full recovery of muscle strength, double-burst stimulation, and spontaneous eye opening, the tracheal tube was removed with no incidence of airway obstruction. She was admitted to the intensive care unit on day 1 postoperatively, where her vital signs were stable. She was extubated on day 1 postoperatively and a percutaneous mini-tracheostomy for pulmonary aspiration was performed. The postoperative course was uneventful. The patient underwent physiotherapy sessions and was discharged on day 6 postoperatively without complications.

One year later, the patient was seen by the emergency department for small bowel occlusion. Blood tests revealed increased CRP [9 mg/L (NV < 1.0), WBC [12 10^9/L (NV 4.0-9.0)] and plasma lactate (3.9 mmol/L). Neurological symptoms were stable and the level of home care was optimal. A second emergency laparotomy was performed, and small bowel ischemia due to ileal obstruction was detected intraoperatively. A small bowel resection was performed and an endoscopic gastrostomy (PEG) tube was placed for enteral nutrition. The same level of protection as in the first surgical procedure was used. The postoperative course was uneventful, and the patient was discharged on day 9 postoperatively with a permanent urinary catheter and PEG tube for enteral nutrition (Table 1).

There are no cases of a patient with GSS undergoing multiple abdominal surgeries under general anesthesia in the literature (according to a search for relevant articles on PubMed and Embase using the terms "GSS" OR "PD" AND "Surgery" (*4*)).

The challenge in the surgical treatment of patients with GSS involves intra- and post-operative anesthesiological risks (*e.g.* airway obstruction, bronchospasm, or pneumonia), surgical risks (*e.g.* immobility, dysphagia, or impaired canalization), and risks of infection. PrP is present in the central nervous system, appendix, and lymphatic tissues and is resistant to inactivation by radiation, heat, or aggressive chemical treatments. Patients with GSS must be managed with specific precautions to prevent infections.

The feasibility of abdominal surgical procedures in patients with GSS cannot be determined based on this single case, despite its favorable outcome. Nonetheless, it indicates that surgery, with adequate caution, can be used to treat this complex condition. In conclusion: *i*) the patient must have family members or caregivers actively participating in post-operative management in the hospital and at home; *ii*) use of disposable equipment is mandatory to avoid the transmission of infection to medical staff; and *iii*) the increased risk of post-operative complications must be taken into account.

Funding: None.

Conflict of interest: The authors have no conflicts of interest to disclose.

References

1. Boellaard JW, Schlote W. Subakute spongiforme Encephalopathie mit multiformer Plaquebildung. "Eigenartige familiär-hereditäre Krankheit des Zentralnervensystems [spino-cerebellare Atrophie mit Demenz, Plaques und plaqueähnlichen Ablagerungen im Klein- und Grossirn" (Gerstmann, Sträussler, Scheinker)] [Subacute spongiform encephalopathy with multiform plaque formation. "Peculiar familial-hereditary disease of CNS [spinocerebellar atrophy with dementia, plaques, and plaque-like deposits in cerebellum and cerebrum" (Gerstmann, Sträussler, Scheinker)] (author's transl)]. Acta Neuropathol. 1980; 49:205-212. (in German)
2. Ghetti B, Piccardo P, Zanusso G. Dominantly inherited prion protein cerebral amyloidoses – A modern view of Gerstmann–Sträussler–Scheinker. Handb Clin Neurol. 2018; 153:243-269.
3. Geschwind MD. Prion Diseases. Continuum (Minneap Minn). 2015; 21 (6 Neuroinfectious Disease): 1612-1638.
4. Nakamura M, Ogata M, Matsuo Y, Sata T. Anesthetic management of a patient with Gerstmann-Sträussler-Scheinker syndrome (mutation of prion protein). Anesth Analg. 2006; 102:1285-1286.
5. Leng B, Sun H, Zhao J, Liu Y, Shen T, Liu W, Liu X, Tan M, Li F, Zhang J, Li Z. Plasma exosomal prion protein levels are correlated with cognitive decline in PD patients. Neurosci Lett. 2020; 723:134866.

Received September 22, 2020; Revised November 30, 2020; Accepted December 12, 2020.

**Address correspondence to:*
Michela Monteleone, General Surgery, San Leopoldo Mandic Hospital, Largo Leopoldo Mandic 1, 23807 Merate LC, Italy.
E-mail: m.monteleone@asst-lecco.it

Released online in J-STAGE as advance publication December 20, 2020.

Letter

DOI: 10.5582/irdr.2020.03084

A novel homozygous variant in exon 10 of the *GALNT3* gene causing hyperphosphatemic familial tumoral calcinosis in a family from North India

Devi Dayal[1,*], Shruti Gupta[2], Rakesh Kumar[1], Radhika Srinivasan[2], Bettina Lorenz-Depiereux[3], Tim M Strom[4]

[1] Endocrinology and Diabetes Unit, Department of Pediatrics, Postgraduate Institute of Medical Education and Research, Chandigarh, India;
[2] Department of Cytology and Gynaecological Pathology, Postgraduate Institute of Medical Education and Research, Chandigarh, India;
[3] Institute of Human Genetics, Helmholtz Zentrum München, Neuherberg, Germany;
[4] Institute of Human Genetics, Technische Universität München, Munich, Germany.

SUMMARY Hyperphosphatemic familial tumoral calcinosis (HFTC) is an extremely rare autosomal recessive disorder caused by variants in the *GALNT3* (N-acetylgalactosaminyltransferase 3), *FGF23* (Fibroblast Growth Factor-23) and *αKL* (α-Klotho) genes, which results in progressive calcification of soft tissues. We describe the case of a 9-year-old girl who presented with recurrent hard nodular swellings on her feet and knees which intermittently discharged chalky white material. Her younger brother also had a similar condition. Both siblings showed hyperphosphatemia, but the parents' biochemical parameters were normal. The histological features of the material aspirated from a skin lesion were consistent with tumoral calcinosis. Sanger sequencing identified a novel homozygous non-synonymous sequence variant in exon 10 of the *GALNT3* gene (NM_004482.3:c.[1681T>A];[1681T>A], NP_004473.2:p.[Cys561Ser];[Cys561Ser] in the proband and her affected brother. The parents were heterozygous carriers for the same sequence variant. In conclusion, we report a new variant in the *GALNT3* gene that caused HFTC in a North Indian family.

Keywords hyperphosphatemic familial tumoral calcinosis, calcinosis cutis, *GALNT3* gene, novel variant, Indian family

Hyperphosphatemic familial tumoral calcinosis (HFTC) is a rare disorder of phosphate metabolism caused by mutations in genes related to Fibroblast Growth Factor-23 (FGF23), which include *FGF23* itself, an FGF23-glycosylating enzyme, N-acetylgalactosaminyltransfer ase 3 (*GALNT3*), and the FGF23 co-receptor α-Klotho (*αKL*) (*1*). The altered gene function decreases FGF23 synthesis or activity and causes increased renal tubular reabsorption of phosphate, thereby increasing blood calcium-phosphate product, which leads to predisposition for soft-tissue calcification (*2*). The most common manifestation of HFTC is calcinosis cutis, which appears clinically as firm, otherwise asymptomatic, white, yellowish or flesh-colored papules, plaques, or nodules. The clinical course is often associated with excretion of chalky material, pain, itching, ulceration, or infection of the lesions (*1*).

Only about 75 patients of genetically confirmed HTFC have been reported (*1*). The majority (about 80%) have mutations in the *GALNT3* gene followed by the *FGF23* gene (about 20%) and the *KL* gene (*1*). Most described patients were of African or Middle East origin, with few cases in Caucasians and Asians (*1-6*). Of about 60 patients reported with the *GALNT3* gene mutations, there is one report of two siblings from India (*7*).

A 9-year-old girl presented with recurrent hard nodular swellings that intermittently discharged chalky white material. The first lesion was noticed at age 4 years on the left knee, which gradually increased in size and ruptured spontaneously. Similar lesions appeared on the right knee and both feet over the next year. At age 5 years, she underwent excision of foot lesions, and was subsequently referred to us for repeated recurrences. There were no dental problems, and pain or redness at the site of lesions. She belonged to a hilly hamlet of the North-Indian state of Himachal Pradesh and was born to non-consanguineous parents. There was no family history of such skin lesions.

Examination showed multiple, small, hard, non-tender masses on the feet and knees, along

Table 1. Results of laboratory investigations of the index patient

Parameter	Patient's value	Reference range
Serum phosphorus	7.5 mg/dL	4.5-5.6 mg/dL
Serum calcium	9.0 mg/dL	9-11 mg/dL
Alkaline phosphatase	216 U/L	50-160 U/L
Serum creatinine	0.5 mg/dL	0.3-0.8 mg/dL
Parathyroid hormone	23.77 pg/mL	10-65 pg/mL
Plasma c-FGF23	2612.7 RU/mL	Upto 125 RU/mL
25-hydroxyvitamin D	11.9 ng/mL	20-100 ng/mL
1, 25-dihydroxyvitamin D	68 nmol/L	50-150 nmol/L
Total leucocyte count	8,800/mm3	4,000-11,000/mm3
ESR	10 mm/hr	0-20 mm/hr
C-reactive protein	1.2 mg/dL	< 0.5 mg/dL
Renal TRP	88%	> 85%
TmP/GFR ratio	3.0 mg/dL	2.9-6.5 mg/dL

c-FGF23, c-terminal fibroblast growth factor 23; ESR, erythrocyte sedimentation rate; TmP/GFR, tubular maximum reabsorption of phosphorus/glomerular filtration rate; TRP, tubular reabsorption of phosphate.

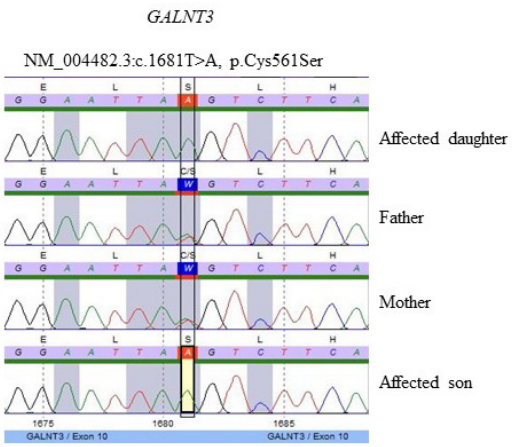


Figure 1. Mutation analysis of the *GALNT3* gene in two affected siblings and their parents. The mutation is indicated above the electropherograms. The affected patients are homozygous for a non synonymous sequence variant in exon 10 of the *GALNT3* gene. The parents are heterozygous carriers.

with an incision scar on the left foot. Her dental, ophthalmological, and systemic examinations were normal. The results of laboratory investigations are shown in Table 1. The younger sibling also showed abnormal serum biochemical parameters (phosphorus 8.1 mg/dL, calcium 8.7 mg/dL, alkaline phosphatase 202 U/L) but normal parathyroid hormone levels (44.94 pg/mL). The parents' biochemistry was normal. Cytological examination of the thick cheese-like material aspirated from one of the skin lesions showed extensive amorphous calcified deposits with granular calcification and clusters of benign epithelial cells of adnexa.

All relevant ethical guidelines have been followed for data collection and reporting. We obtained consent and assent from parents and children respectively, and approval from the Departmental Review Board for reporting data. Genomic DNA was extracted from leucocytes in the peripheral blood of the children and their parents. The 10 coding exons (exons 2-11) of the *GALNT3* gene were amplified by using PCR. Sanger sequencing identified a novel homozygous non-synonymous sequence variant in exon 10 of the *GALNT3* gene (NM_004482.3:c.[1681T>A];[1681T>A], NP_004473.2:p.[Cys561Ser];[Cys561Ser] in both affected siblings (Figure 1). The parents were heterozygous carriers for the same sequence variant (Figure 1). The detected variant is absent from > 251,000 control alleles of the gnomAD browser (*http://gnomad.broadinstitute.org*), which is comprised of exome and genome data from different populations (18,392 East Asian alleles and 30,610 South Asian alleles). This is not published in the literature and has not been reported as clinically relevant in other patients. The c.1681T>A variant is predicted to result in substitution of an evolutionarily highly conserved amino acid that is possibly involved in the formation of an intramolecular disulfid bridge between amino acid Cys561 and Cys574 of the GALNT3 protein. Both children were placed on a low phosphate diet with aluminium hydroxide and sevelamer. The frequency of new lesions decreased and the serum phosphorus concentrations returned to normal levels.

About 40 pathogenic variants in the *GALNT3* gene described so far include missense, nonsense, splice site and frameshift variants in addition to insertions and deletions (*1-7*). Our patients have a missense variant in the *GALNT3* gene while both their parents are heterozygous, and hence, carriers for the disease. Pathogenic variants in the *GALNT3* gene result in a defective GALNT3 protein that is unable to *O*-glycosylate FGF23 (*2*). Therefore, the FGF23 protein is readily cleaved into biologically inactive *N*-terminal and *C*-terminal fragments (c-FGF23). The loss of FGF23 activity leads to hyperphosphatemia typical of HFTC (*1*). The circulating concentrations of c-FGF23 are increased whereas the intact FGF23 (i-FGF23) remains low or inappropriately normal for the level of hyperphosphatemia (*1*).

Differential diagnoses of HFTC include progressive osseous heteroplasia, Cole disease, benign tumoral calcinosis, porphyria cutanea tarda, normophosphatemic FTC, fibrodysplasia ossificans progressiva, iatrogenic tumoral calcinosis and connective tissue disease-associated tumoral calcinosis, which all have normophosphatemia (*1,8*). Very rarely, cutaneous or tendinous xanthomas of homozygous familial hypercholesterolemia needs differentiation from HFTC lesions (*9,10*). Diagnoses of chronic renal failure and pseudohypoparathyroidism were excluded, in our patient, using appropriate biochemical and hormonal investigations.

The phenotypic variability in HFTC is well known (*4*). Family members, in particular siblings, with the same *GALNT3* pathogenic variants, genetic background, and similar biochemical parameters may show marked

variation in disease severity and clinical course (*4*). The younger sibling of our proband showed milder manifestations and disease course.

Acknowledgements

The authors thank the family for their participation, and consent for conducting the laboratory studies and publishing clinical information. We also thank Dr. Manoj Thakur, Department of Orthopedics, IGMC, Shimla for patient care prior to referral.

Funding: None.

Conflict of Interest: The authors have no conflict of interest to disclose.

References

1. Ramnitz MS, Gafni RI, Collins MT. Hyperphosphatemic Familial Tumoral Calcinosis. In: GeneReviews® [Internet] (Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Stephens K, Amemiya A, eds.). Seattle (WA): University of Washington, Seattle; 1993-2020.
2. Kato K, Jeanneau C, Tarp MA, Benet-Pagès A, Lorenz-Depiereux B, Bennett EP, Mandel U, Strom TM, Clausen H. Polypeptide GalNAc-transferase T3 and familial tumoral calcinosis. Secretion of fibroblast growth factor 23 requires O-glycosylation. J Biol Chem. 2006; 281:18370-18377.
3. Benet-Pagès A, Orlik P, Strom TM, Lorenz-Depiereux B. An FGF23 missense mutation causes familial tumoral calcinosis with hyperphosphatemia. Hum Mol Genet. 2005; 14:385-390.
4. Rafaelsen S, Johansson S, Ræder H, Bjerknes R. Long-term clinical outcome and phenotypic variability in hyperphosphatemic familial tumoral calcinosis and hyperphosphatemic hyperostosis syndrome caused by a novel GALNT3 mutation; case report and review of the literature. BMC Genet. 2014; 15:98.
5. Sun L, Zhao L, Du L, Zhang P, Zhang M, Li M, Liu T, Ye L, Tao B, Zhao H, Liu J, Ding X. Identification of two novel mutations in the *GALNT3* gene in a Chinese family with hyperphosphatemic familial tumoral calcinosis. Bone Res. 2016; 4:16038.
6. Kışla Ekinci RM, Gürbüz F, Balcı S, Bişgin A, Taştan M, Yüksel B, Yılmaz M. Hyperphosphatemic familial tumoral calcinosis in two siblings with a novel mutation in *GALNT3* gene: experience from Southern Turkey. J Clin Res Pediatr Endocrinol. 2019; 11:94-99.
7. Joseph L, Hing SN, Presneau N, O'Donnell P, Diss T, Idowu BD, Joseph S, Flanagan AM, Delaney D. Familial tumoral calcinosis and hyperostosis-hyperphosphataemia syndrome are different manifestations of the same disease: novel missense mutations in GALNT3. Skeletal Radiol. 2010; 39:63-68.
8. Randhawa MS, Varma TH, Dayal D. Benign calcinosis cutis. Turk Pediatri Ars. 2018; 53:267-268.
9. Tandon S, Sardana K, Malhotra P, Singh J. Multiple asymptomatic juxta-articular nodules mimicking tuberous-xanthoma – a unusual presentation of tophaceous gout. J Cutan Aesthet Surg. 2017; 10:223-225.
10. Dayal D, Seetharaman K, Bhunwal S, Jain N. Long-term use of a combination of atorvastatin and ezetimibe in children with homozygous familial hypercholesterolemia. Int J Contemp Pediatr. 2018; 5:275-277.

Received July 24, 2020; Revised October 6, 2020; Accepted October 20, 2020.

**Address correspondence to:*
Devi Dayal, Endocrinology and Diabetes Unit, Department of Pediatrics, 3108, Level III, Advanced Pediatrics Center, Postgraduate Institute of Medical Education and Research, Chandigarh-160012, India.
E-mail: drdevidayal@gmail.com

Released online in J-STAGE as advance publication November 20, 2020.

Letter

DOI: 10.5582/irdr.2020.03086

Pre-Paget cells express a Paget cell marker before losing a keratinocyte marker

Allen A. Smith*

Barry University School of Podiatric Medicine, Miami Shores, Florida, USA.

SUMMARY Extramammary Paget's disease (EMPD) is a cancer of the anogenital epithelium. Its origin has been variously attributed to keratinocytes or to Toker cells. Slides of 3 advanced cases of EMPD were incubated with trypsin to retrieve antigens. The slides were then stained with rabbit polyclonal anti-carcinoembryonic antigen to mark Paget cells and mouse monoclonal anti-cytokeratin 10 to mark keratinocytes. Several cells in each case stained with both the Paget cell marker and the keratinocyte marker. The presence of cells with both markers shows that Paget cells originate from keratinocytes. The presence of pre-Paget cells in advanced cases of EMPD shows that Paget cells are continuously recruited from keratinocytes.

Keywords extramammary Paget's disease, EMPD, carcinoembryonic antigen, cytokeratin 10

Extramammary Paget's disease (EMPD) is a cancer that arises in the epidermis of the anogenital region and expands and migrates in the epidermis before invading the dermis (*1*). Its incidence has increased during the last generation (*2*).

Toker cells, which resemble Paget cells, have been suggested as the source of EMPD (*3*), but they are not seen in most cases of EMPD (*4*). There have been several observations of a few cells with the morphology of keratinocytes that do express a Paget cell marker in cases of EMPD (*5,6*). None of these observations provided histochemical evidence that the rare cells with Paget cell markers were keratinocytes.

Cytokeratin 10 (CK10) is a keratinocyte marker which has not been observed in Paget cells (*7*). Carcinoembryonic antigen (CEA), recently renamed CD66e, is a Paget cell marker which is never expressed in normal epidermis (*8*).

Mounted formalin-fixed paraffin-embedded sections of 3 cases of EMPD, 2 in the labium majus and 1 in the hood of the clitoris, were obtained from the Cooperative Human Tissue Network. Antigens were retrieved by exposure to 0.05% trypsin for 20 min at 37oC. Nonspecific antibodies were blocked by 30 min incubation in 2.5% normal horse serum. The tissue was incubated overnight in a 1:1 mixture of 1/20 mouse monoclonal anti-CK10 (Genetex GTX21421) and 1/100 rabbit polyclonal anti-CD66e (GTX108732) in PBS. The tissue was stained with Duett conjugated secondary antibody mixture (Vector Labs MP-7724), DAB, and Vector Red.

There were many areas of confluent Paget cells, but there were also areas of morphologically normal keratinocytes. All keratinocytes expressed CK10. Almost all Paget cells expressed carcinoembryonic antigen. A few morphologically normal keratinocytes expressed both CK10 and CEA (Figure 1 and Figure 2). These cells are so few in number that they are easily missed (Figure 1). Rarely, cells expressing both CK10 and CEA were too close to round for normal keratinocytes (Figure 3).

The presence of cells expressing both CK10 and CEA in these cases proves that at least some cases of EMPD originate from keratinocytes. Cells expressing both markers must be pre-Paget cells. This conclusion is reinforced by the presence of rare cells expressing both markers that are intermediate in shape between keratinocytes and Paget cells (Figure 3).

The presence of pre-Paget cells in advanced cases of EMPD shows that malignant changes occur repeatedly in EMPD rather than in just a single progenitor cell. This can lead to multfocal extramammary Paget's disease (*1,9*).

While the expression of carcinoembryonic antigen may not be the first step in the malignant transformation of a keratinocyte in EMPD, it seems to be an essential step (*10*). The fact that most cells expressing both CK10 and CEA have the morphology of keratinocytes suggests that the expression of CEA is an early step. The expression of carcinoembryonic antigen probably

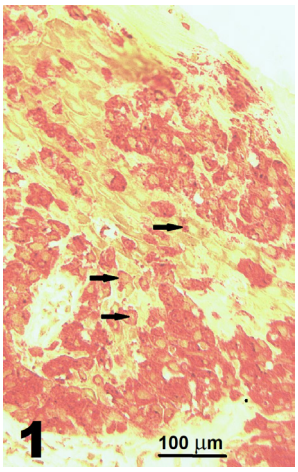


Figure 1. EMPD of the labium majus. Two pre-Paget cells (solid arrows) are present in the lower epidermis. Cytokeratin 10 (CK 10) stained with diaminobenzidine (DAB); carcinoembryonic antigen (CEA) stained with Vector Red.

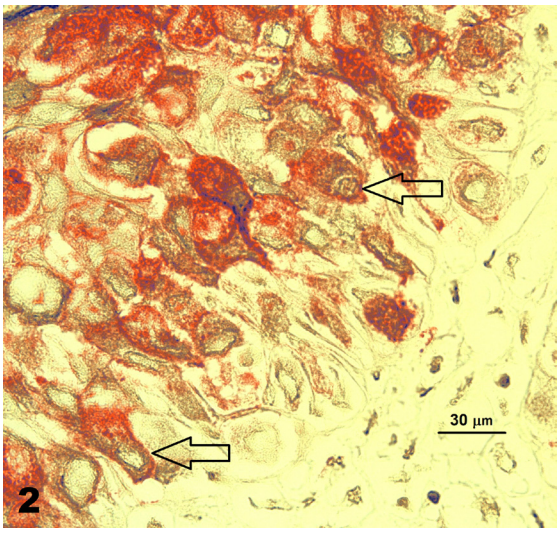


Figure 2. EMPD of the clitoridal hood. One pre-Paget cell is in the basal epidermis; a second is in the stratum spinosum (hollow arrows). CK10 stained with DAB; CEA stained with Vector Red.

blocks differentiation of keratinocytes just as it blocks differentiation of myoblasts (*10*).

Funding: None.

Conflict of Interest: The author has no conflict of interest to disclose.

References

1. Delport ES. Extramammary Paget's disease of the vulva: An annotated review of the literature. Austral J Dermatol. 2013; 54:9-21.
2. Mai R, Zhou S, Zhou S, Zhong W, Hong L, Wang Y, Lu S, Pan J, Huang Y, Su M, Crawford R, Zhou Y, Zhang G. Transcriptome analyses reveal FOXA1 dysregulation in mammary and extramammary Paget's disease. Hum Pathol. 2018; 77:152-158.
3. Wilman JH, Golitz LE, Fitzpatrick JF. Vulvar clear cells of Toker: precursors of extramammary Paget's disease. Am J Dermatopathol. 2005; 27:185-188.
4. Liegl-Atzwanger B, Moinfar F. "Toker cells" as origin of Paget's disease: fact or Fiction? Histopathology. 2008; 52:891-892.
5. Bussolati G, Pich A. Mammary and extramammary Paget's disease. An immunocytochemical study. Am J Pathol. 1975; 80:117-127.
6. Smith AA. Pre-Paget cells: evidence of keratinocyte origin of extramammary Paget's disease. Intract Rare Dis Res. 2019; 8:203-205.
7. Moll I, Moll R. Cells of extramammary Paget's disease express cytokeratins different from those of epidermal cells. J Invest Dermatol. 1985; 84:3-8.
8. Liegl B, Liegl S, Gogg-Kammerer M, Tessaro B, Horn LC, Moinfar F. Mammary and extramammary Paget's disease: an immunohistochemical study of 83 cases. Histopathology. 2007; 50:439-447.
9. Leelavathi M, Norazirah MN, Nur Amira AP. Multiple concurrent primary extramammary Paget's disease. Malay Fam Physician. 2016; 11:18-21.
10. Screaton RA, Penn LZ, Stanners CP. Carcinoembryonic antigen, a human tumor marker, cooperates with myc and bcl-2 in cellular transformation. J Cell Biol. 1997; 137: 939-952.

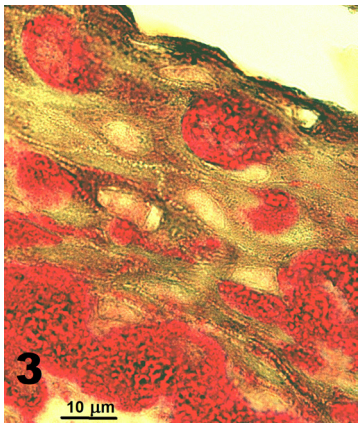


Figure 3. EMPD of the labium majus. A pre-Paget cell in the upper epidermis has almost completed its transition to a Paget cell. CK10 stained with DAB; CEA stained with Vector Red.

Received July 31, 2020; Revised October 10, 2020; Accepted October 20, 2020.

**Address correspondence to:*
Allen A. Smith, Barry University School of Podiatric Medicine, 11300 NE 2nd Ave., Miami Shores, FL 33161, USA.
E-mail: asmith@barry.edu

Released online in J-STAGE as advance publication November 20, 2020.

Letter

DOI: 10.5582/irdr.2020.03091

Establishing a rare diseases center: Experiences from Western China

Li Gong[1], Qian He[2,*]

[1] Rare Diseases Center, West China Hospital, Sichuan University, Chengdu, Sichuan, China;
[2] Department of Outpatient, West China Hospital, Sichuan University, Chengdu, Sichuan, China.

SUMMARY Rare diseases pose unique challenges to health care delivery. In August 2016, the West China Hospital of Sichuan University (WCHSU) established a rare diseases center. This center has created a multidisciplinary team of rare disease experts. The center provides expedited pathways online and offline for patients with rare diseases to save them time and money, to improve their experience, and to increase the hospital's efficiency. At the same time, the center regularly organizes public education campaigns and it offers free consultations to enhance awareness of rare diseases. Establishment of the rare disease alliance and facilitation of 5G-based remote multi-disciplinary consultations will help to improve the level of diagnosis and treatment and to solve problems with diagnosis and treatment encountered by local patients with rare diseases. WCHSU's rare diseases center has been feasible, acceptable, and effective in Western China and it should benefit patients, doctors, and hospitals. The center should lead to significant improvements in treatment for patients with rare diseases. The successful establishment of a rare diseases center here may be a useful reference for other parts of the world.

Keywords rare diseases, center, experiences, China, platform

Rare diseases refer to diseases with a very low prevalence. There are more than 7,000 rare diseases recognized internationally, accounting for about 10% of human diseases; 80% of rare diseases are genetic, and 50 to 60% occur in childhood (*1-3*). Because of the large number of people affected, patients with rare diseases are actually "not rare" in China (*4*). Due to the complex etiology, heterogeneous symptoms, and the limited forms of examinations, rare diseases are often undiagnosed and there are few treatment options (*5*). Other challenges include a lack of information and resources, the financial cost of care, and difficulty in accessing appropriate medical expertise, which is compounded by a lack of specialist training programs for medical professionals (*6*). The current situation needs to be improved urgently (*7*).

In August 2016, the West China Hospital of Sichuan University (WCHSU) established a rare diseases center in order to provide better medical care for patients with rare diseases in Southwest China and better treatments and therapies for patients with rare diseases in China. WCHSU is a prestigious and well-known medical center located in the City of Chengdu, Sichuan Province. This is the first medical facility to establish a rare diseases center among hospitals in China. The center has created a team of multidisciplinary rare diseases experts, devised procedures for center operations, and provided patients with rare diseases with an expedited pathway for faster diagnosis and treatment. Specialists specifically provide patients with one-stop care.

In February 2018, the center officially launched an online rare disease platform to disseminate information about rare diseases and to facilitate remote diagnosis and treatment – "Huaxi Rare Diseases," the center's official WeChat account. This is the first online platform built specifically for patients with rare diseases in Chinese hospitals. The platform's features include submission of applications for diagnosis and treatment of rare diseases, online consultations, introductions to experts in various departments, a rare disease encyclopedia, and special medical treatments. Patients with rare diseases can submit diagnosis and treatment applications on the platform. If the patient's application is approved, the center will make an appointment with a specialist clinic for the patient. In addition, doctors also provide patients with online outpatient diagnosis and treatment services through video and audio online so that patients with rare diseases can see a doctor without leaving home.

In November 2018, the center took the lead by establishing the Rare Disease Committee of the

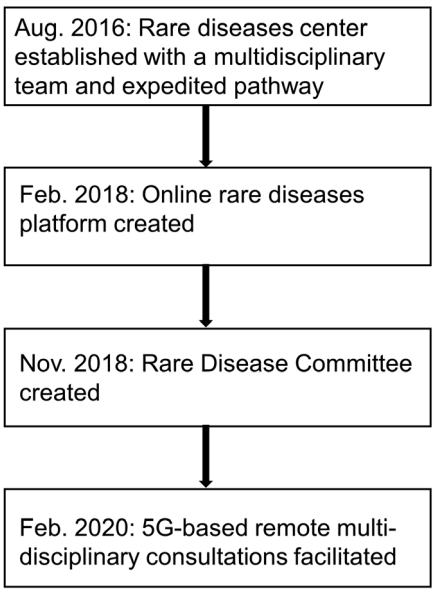


Figure 1. Flowchart depicting establishment of the rare diseases center.

Sichuan Medical Association. The committee regularly provides continuing education to doctors to promote and encourage the spread of new technologies, new drugs, and new findings related to the diagnosis and treatment of rare diseases. The committee also regularly organizes public education campaigns in order to disseminate medical information about rare diseases, to improve the public's awareness and level of self-care, to standardize the treatment of patients with rare diseases, to provide more information to patients with rare diseases and their families, to encourage a positive attitude among patients with rare diseases, and to improve their quality of life.

In February 2020, the hospital launched a new 5G web-based, real-time video telemedicine system for consultations. A multidisciplinary team deals with cases of rare diseases to serve patients and to help improve the level of diagnosis and treatment. The hospital plans to coordinate with hospitals at all levels to regularly conduct remote rare disease consultations and case discussions.

From August 2016 to February 2020, the center helped a total of 1,185 patients with rare diseases on-site, and it provided assistance to 2,169 including patient registration and admission to hospital. Two campaigns online reached more than 60,000 people. The WeChat account has 7,531 followers, it has published a total of 81 messages on rare diseases, and its messages have been read 100,209 times, with an average of 1,237 times per article. The online platform has received 866 patient applications, including 431 patients with rare diseases and 435 patients who do not meet the center's requirements. Of the 431 patients with rare diseases who applied for diagnosis and treatment online, 306 (71.0%) visited offline. Submitting an application *via* the platform to a consultation offline takes 0-7 days, and the average time to a consultation is 4.31 ± 2.18 days. The rare disease committee has organized three large-scale academic conferences on rare diseases to describe the latest advances in clinical and scientific research on rare diseases to more than 3,000 people, and it has organized more than 80 public events and free clinic visits. In February 2020, a survey of 100 patients with rare diseases and 50 doctors at primary hospitals and this hospital found that all of the respondents were 100% satisfied with the center.

Funding: This study was supported by the 1.3.5 project for disciplines of excellence, West China Hospital, Sichuan University (Grant/Award Number: ZYJC18003). The authors wish to thank Prof. Yi Liu for his valuable suggestions and help editing this manuscript.

Conflict of interest: The authors have no conflicts of interest to disclose.

References

1. EURORDIS. About rare diseases. *http://www.eurordis.org/about-rare-diseases* (accessed January 6, 2020).
2. EURORDIS. Rare Diseases: Understanding this public health priority. *http://www.eurordis.org/publication/rare-diseases-understanding-public-health-priority* (accessed January 6, 2020)
3. Jin X, Chen L. Orphan drug development in China - Turning challenges into opportunities. Intractable Rare Dis Res. 2016; 5:308-313.
4. Cui Y, Han J. Defining rare diseases in China. Intractable Rare Dis Res. 2017; 6:148-149.
5. Song P, He J, Li F, Jin C. Innovative measures to combat rare diseases in China: The national rare diseases registry system, larger-scale clinical cohort studies, and studies in combination with precision medicine research. Intractable Rare Dis Res. 2017; 6:1-5.
6. The Lancet Diabetes Endocrinology. Spotlight on rare diseases. Lancet Diabetes Endocrinol. 2019; 7:75.
7. Ferreira CR. The burden of rare diseases. Am J Med Genet A. 2019; 179:885-892.

Received on August 17, 2020; Revised on October 20, 2020; Accepted on December 11, 2020.

**Address correspondence to:*
Qian He, Department of Outpatient, West China Hospital, Sichuan University, No.37 Guo Xue Xiang, Chengdu 610041, Sichuan, China.
E-mail: heqian@wchscu.cn

Released online in J-STAGE as advance publication December 20, 2020.

IRDR
Intractable & Rare Diseases Research

Guide for Authors

1. Scope of Articles

Intractable & Rare Diseases Research (Print ISSN 2186-3644, Online ISSN 2186-361X) 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

Original Articles should be well-documented, novel, and significant to the field as a whole. An Original Article should be arranged into the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References. Original articles should not exceed 5,000 words in length (excluding references) and should be limited to a maximum of 50 references. Articles may contain a maximum of 10 figures and/or tables. Supplementary Data are permitted but should be limited to information that is not essential to the general understanding of the research presented in the main text, such as unaltered blots and source data as well as other file types.

Brief Reports definitively documenting either experimental results or informative clinical observations will be considered for publication in this category. Brief Reports are not intended for publication of incomplete or preliminary findings. Brief Reports should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 4 figures and/or tables and 30 references. A Brief Report contains the same sections as an Original Article, but the Results and Discussion sections should be combined.

Reviews should present a full and up-to-date account of recent developments within an area of research. Normally, reviews should not exceed 8,000 words in length (excluding references) and should be limited to a maximum of a maximum of 10 figures and/or tables and 100 references. Mini reviews are also accepted, which should not exceed 4,000 words in length (excluding references) and should be limited to a maximum of 5 figures and/or tables and 50 references.

Policy Forum articles discuss research and policy issues in areas related to life science such as public health, the medical care system, and social science and may address governmental issues at district, national, and international levels of discourse. Policy Forum articles should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 5 figures and/or tables and 30 references.

Case Reports should be detailed reports of the symptoms, signs, diagnosis, treatment, and follow-up of an individual patient. Case reports may contain a demographic profile of the patient but usually describe an unusual or novel occurrence. Unreported or unusual side effects or adverse interactions involving medications will also be considered. Case Reports should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 5 figures and/or tables and 30 references.

Communications are short, timely pieces that spotlight new research findings or policy issues of interest to the field of global health and medical practice that are of immediate importance. Depending on their content, Communications will be published as "Comments" or "Correspondence". Communications should not exceed 1,500 words in length (excluding references) and should be limited to a maximum of 2 figures and/or tables and 20 references.

Editorials are short, invited opinion pieces that discuss an issue of immediate importance to the fields of global health, medical practice, and basic science oriented for clinical application. Editorials should not exceed 1,000 words in length (excluding references) and should be limited to a maximum of 10 references. Editorials may contain one figure or table.

News articles should report the latest events in health sciences and medical research from around the world. News should not exceed 500 words in length.

Letters should present considered opinions in response to articles published in *Intractable & Rare Diseases Research* in the last 6 months or issues of general interest. Letters should not exceed 800 words in length and may contain a maximum of 10 references. Letters may contain one figure or table.

3. Editorial Policies

For publishing and ethical standards, *Intractable & Rare Diseases Research* follows the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (*http://www.icmje.org/recommendations*) issued by the International Committee of Medical Journal Editors (ICMJE), and the Principles of Transparency and Best Practice in Scholarly Publishing (*https://doaj.org/bestpractice*) jointly issued by the Committee on Publication Ethics (COPE), the Directory of Open Access Journals (DOAJ), the Open Access Scholarly Publishers Association (OASPA), and the World Association of Medical Editors (WAME).

Intractable & Rare Diseases Research will perform an especially prompt review to encourage innovative work. All original research will be subjected to a rigorous standard of peer review and will be edited by experienced copy editors to the highest standards.

Ethics: *Intractable & Rare Diseases Research* requires that authors of reports of investigations in humans or animals indicate that those studies were formally approved by a relevant ethics committee or review board. For research involving human experiments, a statement that the participants gave informed consent before taking part (or a statement that it was not required and why) should be indicated. Authors should also state that the study conformed to the provisions of the Declaration of Helsinki (as revised in 2013). When reporting experiments on animals, authors should indicate whether

the institutional and national guide for the care and use of laboratory animals was followed.

Conflict of Interest: All authors are required to disclose any actual or potential conflict of interest including financial interests or relationships with other people or organizations that might raise questions of bias in the work reported. If no conflict of interest exists for each author, please state "There is no conflict of interest to disclose".

Submission Declaration: When a manuscript is considered for submission to *Intractable & Rare Diseases Research*, the authors should confirm that 1) no part of this manuscript is currently under consideration for publication elsewhere; 2) this manuscript does not contain the same information in whole or in part as manuscripts that have been published, accepted, or are under review elsewhere, except in the form of an abstract, a letter to the editor, or part of a published lecture or academic thesis; 3) authorization for publication has been obtained from the authors' employer or institution; and 4) all contributing authors have agreed to submit this manuscript.

Cover Letter: The manuscript must be accompanied by a cover letter prepared by the corresponding author on behalf of all authors. The letter should indicate the basic findings of the work and their significance. The letter should also include a statement affirming that all authors concur with the submission and that the material submitted for publication has not been published previously or is not under consideration for publication elsewhere. The cover letter should be submitted in PDF format. For example of Cover Letter, please visit: Download Centre (*https://www.irdrjournal.com/downcentre*).

Copyright: When a manuscript is accepted for publication in *Intractable & Rare Diseases Research*, the transfer of copyright is necessary. A JOURNAL PUBLISHING AGREEMENT (JPA) form will be e-mailed to the authors by the Editorial Office and must be returned by the authors as a scan. Only forms with a hand-written signature are accepted. This copyright will ensure the widest possible dissemination of information. Please note that your manuscript will not proceed to the next step in publication until the JPA Form is received. In addition, if excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article.

Peer Review: *Intractable & Rare Diseases Research* uses single-blind peer review, which means that reviewers know the names of the authors, but the authors do not know who reviewed their manuscript. The external peer review is performed for research articles by at least two reviewers, and sometimes the opinions of more reviewers are sought. Peer reviewers are selected based on their expertise and ability to provide high quality, constructive, and fair reviews. For research manuscripts, the editors may, in addition, seek the opinion of a statistical reviewer. Consideration for publication is based on the article's originality, novelty, and scientific soundness, and the appropriateness of its analysis.

Suggested Reviewers: A list of up to 3 reviewers who are qualified to assess the scientific merit of the study is welcomed. Reviewer information including names, affiliations, addresses, and e-mail should be provided at the same time the manuscript is submitted online. Please do not suggest reviewers with known conflicts of interest, including participants or anyone with a stake in the proposed research; anyone from the same institution; former students, advisors, or research collaborators (within the last three years); or close personal contacts. Please note that the Editor-in-Chief may accept one or more of the proposed reviewers or may request a review by other qualified persons.

Language Editing: Manuscripts prepared by authors whose native language is not English should have their work proofread by a native English speaker before submission. If not, this might delay the publication of your manuscript in *Intractable & Rare Diseases Research*.

The Editing Support Organization can provide English proofreading, Japanese-English translation, and Chinese-English translation services to authors who want to publish in *Intractable & Rare Diseases Research* and need assistance before submitting a manuscript. Authors can visit this organization directly at *http://www.iacmhr.com/iac-eso/support.php?lang=en*. IAC-ESO was established to facilitate manuscript preparation by researchers whose native language is not English and to help edit works intended for international academic journals.

4. Manuscript Preparation

Manuscripts are suggested to be prepared in accordance with the "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals", as presented at *http://www.ICMJE.org.*

Manuscripts should be written in clear, grammatically correct English and submitted as a Microsoft Word file in a single-column format. Manuscripts must be paginated and typed in 12-point Times New Roman font with 24-point line spacing. Please do not embed figures in the text. Abbreviations should be used as little as possible and should be explained at first mention unless the term is a well-known abbreviation (*e.g.* DNA). Single words should not be abbreviated.

Title page: The title page must include 1) the title of the paper (Please note the title should be short, informative, and contain the major key words); 2) full name(s) and affiliation(s) of the author(s), 3) abbreviated names of the author(s), 4) full name, mailing address, telephone/fax numbers, and e-mail address of the corresponding author; and 5) conflicts of interest (if you have an actual or potential conflict of interest to disclose, it must be included as a footnote on the title page of the manuscript; if no conflict of interest exists for each author, please state "There is no conflict of interest to disclose"). Please visit Download Centre and refer to the title page of the manuscript sample.

Abstract: The abstract should briefly state the purpose of the study, methods, main findings, and conclusions. For articles that are Original Articles, Brief Reports, Reviews, Policy Forum articles, or Case Report, a one-paragraph abstract consisting of no more than 250 words must be included in the manuscript. For Communications, Editorials, News, or Letters, a brief summary of main content in 150 words or fewer should be included in the manuscript. Abbreviations must be kept to a minimum and non-standard abbreviations explained in brackets at first mention. References should be avoided in the abstract. Three to six key words or phrases that do not occur in the title should be included in the Abstract page.

Introduction: The introduction should be a concise statement of the basis for the study and its scientific context.

Materials and Methods: The description should be brief but with sufficient detail to enable others to reproduce the experiments. Procedures that have been published previously should not be described in detail but appropriate references should simply be cited. Only new and significant modifications of previously published procedures require complete description. Names of products and manufacturers with their locations (city and state/country) should be given and sources of animals and cell lines should always be indicated. All clinical investigations must have been conducted in accordance with Declaration of Helsinki principles. All human and animal studies must have been approved by the appropriate institutional review board(s) and a specific declaration of approval must be made within this section.

Results: The description of the experimental results should be succinct but in sufficient detail to allow the experiments to be analyzed and interpreted by an independent reader. If necessary, subheadings may be used for an orderly presentation. All figures and tables must be referred to in the text.

Discussion: The data should be interpreted concisely without repeating material already presented in the Results section. Speculation is permissible, but it must be well-founded, and discussion of the wider implications of the findings is encouraged. Conclusions derived from the study should be included in this section.

Acknowledgments: All funding sources should be credited in the Acknowledgments section. In addition, people who contributed to the work but who do not meet the criteria for authors should be listed along with their contributions.

References: References should be numbered in the order in which they appear in the text. Citing of unpublished results, personal communications, conference abstracts, and theses in the reference list is not recommended but these sources may be mentioned in the text. In the reference list, cite the names of all authors when there are fifteen or fewer authors; if there are sixteen or more authors, list the first three followed by *et al*. Names of journals should be abbreviated in the style used in PubMed. Authors are responsible for the accuracy of the references. The EndNote Style of *Intractable & Rare Diseases Research* could be downloaded at **EndNote** (*https://www.irdrjournal.com/examples/Intractable_Rare_Diseases_Research.ens*).

Examples are given below:

Example 1 (Sample journal reference):

Inagaki Y, Tang W, Zhang L, Du GH, Xu WF, Kokudo N. Novel aminopeptidase N (APN/CD13) inhibitor 24F can suppress invasion of hepatocellular carcinoma cells as well as angiogenesis. Biosci Trends. 2010; 4:56-60.

Example 2 (Sample journal reference with more than 15 authors):

Darby S, Hill D, Auvinen A, *et al*. Radon in homes and risk of lung cancer: Collaborative analysis of individual data from 13 European case-control studies. BMJ. 2005; 330:223.

Example 3 (Sample book reference):
Shalev AY. Post-traumatic stress disorder: Diagnosis, history and life course. In: Post-traumatic Stress Disorder, Diagnosis, Management and Treatment (Nutt DJ, Davidson JR, Zohar J, eds.). Martin Dunitz, London, UK, 2000; pp. 1-15.

Example 4 (Sample web page reference):

World Health Organization. The World Health Report 2008 – primary health care: Now more than ever. *http://www.who.int/whr/2008/whr08_en.pdf* (accessed September 23, 2010).

Tables: All tables should be prepared in Microsoft Word or Excel and should be arranged at the end of the manuscript after the References section. Please note that tables should not in image format. All tables should have a concise title and should be numbered consecutively with Arabic numerals. If necessary, additional information should be given below the table.

Figure Legend: The figure legend should be typed on a separate page of the main manuscript and should include a short title and explanation. The legend should be concise but comprehensive and should be understood without referring to the text. Symbols used in figures must be explained. Any individually labeled figure parts or panels (A, B, *etc*.) should be specifically described by part name within the legend.

Figure Preparation: All figures should be clear and cited in numerical order in the text. Figures must fit a one- or two-column format on the journal page: 8.3 cm (3.3 in.) wide for a single column, 17.3 cm (6.8 in.) wide for a double column; maximum height: 24.0 cm (9.5 in.). Please make sure that the symbols and numbers appeared in the figures should be clear. Please make sure that artwork files are in an acceptable format (TIFF or JPEG) at minimum resolution (600 dpi for illustrations, graphs, and annotated artwork, and 300 dpi for micrographs and photographs). Please provide all figures as separate files. Please note that low-resolution images are one of the leading causes of article resubmission and schedule delays.

Units and Symbols: Units and symbols conforming to the International System of Units (SI) should be used for physicochemical quantities. Solidus notation (*e.g*. mg/kg, mg/mL, mol/mm^2/min) should be used. Please refer to the SI Guide *www.bipm.org/en/si/* for standard units.

Supplemental data: Supplemental data might be useful for supporting and enhancing your scientific research and *Intractable & Rare Diseases Research* accepts the submission of these materials which will be only published online alongside the electronic version of your article. Supplemental files (figures, tables, and other text materials) should be prepared according to the above guidelines, numbered in Arabic numerals (*e.g*., Figure S1, Figure S2, and Table S1, Table S2) and referred to in the text. All figures and tables should have titles and legends. All figure legends, tables and supplemental text materials should be placed at the end of the paper. Please note all of these supplemental data should be provided at the time of initial submission and note that the editors reserve the right to limit the size and length of Supplemental Data.

5. Submission Checklist

The Submission Checklist will be useful during the final checking of a manuscript prior to sending it to *Intractable & Rare Diseases Research* for review. Please visit Download Centre and download the Submission Checklist file.

6. Online Submission

Manuscripts should be submitted to *Intractable & Rare Diseases Research* online at *https://www.irdrjournal.com*. The manuscript file should be smaller than 5 MB in size. If for any reason you are unable to submit a file online, please contact the Editorial Office by e-mail at *office@irdrjournal.com*

7. Accepted Manuscripts

Proofs: Galley proofs in PDF format will be sent to the corresponding author *via* e-mail. Corrections must be returned to the editor (*office@irdrjournal.com*) within 3 working days.

Offprints: Authors will be provided with electronic offprints of their article. Paper offprints can be ordered at prices quoted on the order form that accompanies the proofs.

Page Charge: No page charges will be levied to authors for the publication of their article except for reprints.

Misconduct: *Intractable & Rare Diseases Research* takes seriously all allegations of potential misconduct and adhere to the ICMJE Guideline (*http://www.icmje.org/recommendations*) and COPE Guideline (*http://publicationethics.org/files/Code_of_conduct_for_journal_editors.pdf*). In cases of suspected research or publication misconduct, it may be necessary for the Editor or Publisher to contact and share submission details with third parties including authors' institutions and ethics committees. The corrections, retractions, or editorial expressions of concern will be performed in line with above guidelines.

(As of June 2020)

Intractable & Rare Diseases Research
Editorial and Head Office
Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan.
E-mail: office@irdrjournal.com