ISSN 2186-3644 Online ISSN 2186-361X

IRDR

Intractable & Rare Diseases Research

Volume 1, Number 4
November, 2012

IRCA BSSA

IACMHR

www.irdrjournal.com

IRDR
Intractable & Rare Diseases Research

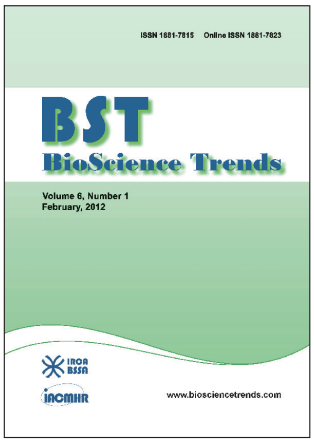
ISSN: 2186-3644
Online ISSN: 2186-361X
CODEN: IRDRA3
Issues/Year: 4
Language: English
Publisher: IACMHR Co., Ltd.

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

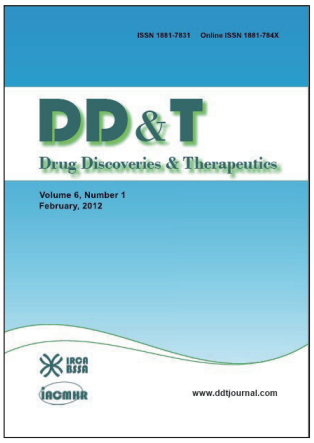
Intractable & Rare Diseases Research devotes to publishing the latest and most significant research in intractable and rare diseases. Articles cover all aspects of intractable and rare diseases research such as molecular biology, genetics, clinical diagnosis, prevention and treatment, epidemiology, health 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, News, and Letters on all aspects of the field of intractable and rare diseases research. All contributions should seek to promote international collaboration.

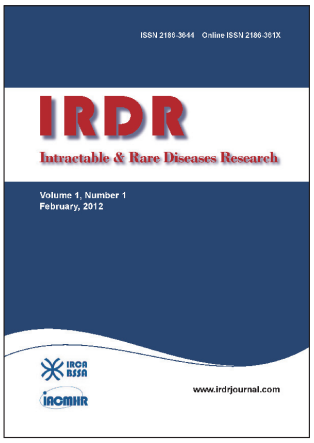
IRCA-BSSA Group Journals



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

Tel: +81-3-5840-9968, Fax: +81-3-5840-9969
E-mail: office@irdrjournal.com
URL: www.irdrjournal.com

Editorial Board

CONTENTS

Volume 1, Number 4, 2012

Policy Forum

Reviews

Brief Report

Original Article

Case Report

CONTENTS (*Continued*)

Policy Forum

DOI: 10.5582/irdr.2012.v1.4.139

Access to orphan drugs in the Middle East: Challenge and perspective

Ziyad S. Almalki[1], Abdullah K. Alahmari[1], Jeff J. Guo[1,*], Christina M.L. Kelton[2]

[1] *The James L. Winkle College of Pharmacy, University of Cincinnati Academic Health Center, Cincinnati, OH, USA;*
[2] *The Carl H. Lindner College of Business, University of Cincinnati, Cincinnati, OH, USA.*

Summary **An orphan drug is a drug developed specifically to treat a rare medical condition. With a combined population of less than 400 million, about 2.8 million patients are estimated to be suffering from a rare disease in the Middle East. Some disorders such as hemoglobinopathy, glucose-6-phosphate dehydrogenase deficiency, autosomal recessive syndromes, and several metabolic disorders have a presence throughout the Middle East. In order to promote the treatment of these diseases, Middle Eastern governments need to facilitate education and training of healthcare personnel; develop and execute a method for obtaining and paying for orphan drugs; and, finally, provide tax, marketing, and other incentives to domestic and international firms to develop drugs specifically for the diseases of most importance to Middle Eastern patients.**

Keywords: Orphan drug, rare disease, genetic disorder, Middle East

1. Introduction

An orphan drug is a drug developed specifically to treat a rare medical condition (*1*). Because the high cost of drug development tends to discourage pharmaceutical companies from developing products for very small populations of patients, public-sector involvement becomes critical to the success of orphan-drug markets. Legislation has been implemented by the United States, the European Union, Japan, Singapore, Taiwan, South Korea, and Australia that offers subsidies and other incentives to encourage the development of orphan drugs (*2*). Some companies, such as Genzyme, acquired by Sanofi-Aventis in 2011, have even thrived under such legislation, focusing their efforts on developing treatments for rare diseases as a profitable business strategy (*3*). Myozyme (alglucosidase alfa, recombinant human GAA) and Lumizyme (alglucosidase alfa), both Genzyme products and the first two therapies available for Pompe disease, were approved as orphan drugs by the U.S. Food and Drug Administration (FDA) in 2006 and in 2010, respectively (*4,5*). They significantly improve survival for those patients suffering from this rare condition, affecting 5,000-10,000 people worldwide (*6*). Other recently approved orphan drugs include Glaxo's Lexiva (fosamprenavir) for HIV infection, Genzyme's Fabrazyme (agalsidase beta) for Fabry disease, and Novartis's Visudyne (verteporfin) for age-related macular degeneration (*2*).

As high-quality healthcare becomes a growing priority in developing countries, it is not surprising to see a rising interest in rare diseases and potential treatments in those countries as well. Public awareness of rare diseases is growing in China, where at least 10 million people (out of over 1.3 billion), *i.e.*, approximately 0.7% of the Chinese population, are estimated to be living with osteogenesis imperfecta, Fabry disease, hemophilia A and B, albinism, acromegaly, and other rare conditions (*7*). At this point in time, however, Chinese patients do not have good access to orphan drugs, nor are Chinese pharmaceutical companies participating in new orphan drug development (*7*). Meanwhile, rare-disease patients and their advocates in a number of Middle Eastern countries are finding themselves in a similar situation to those in China. As living standards improve in the Middle East, healthcare providers face higher expectations for better quality healthcare products and services (*8*). With a combined population of less than 400 million, about

**Address correspondence to:*
Dr. Jeff J. Guo, The James L. Winkle College of Pharmacy, University of Cincinnati Academic Health Center, Cincinnati, OH 45267, USA.
E-mail: jeff.guo@uc.edu

2.8 million patients are estimated to be suffering from a rare disease in the Middle East (*7,9*).

2. Rare diseases in the Middle East

The Middle East, in its "narrow" definition, consists of 16 countries (in declining order by population: Egypt, Iran, Turkey, Iraq, Saudi Arabia, Yemen, Syria, United Arab Emirates or UAE, Israel, Jordan, Lebanon, Oman, Kuwait, Qatar, Bahrain, and Cyprus) plus the Palestinian territories of the West Bank and the Gaza Strip. Using the 0.7% prevalence rate for China and the United Nations' population estimates for countries in the Middle East, estimated numbers of patients with rare diseases are shown for the Middle Eastern countries in Figure 1.

The population of the region is characterized by large family size, older maternal and paternal age, and a high rate (25-60%) of consanguineous marriages (*10*). Hence, the risk for genetic disorders may be higher than in other regions of the world. Indeed, such disorders account for the majority of rare diseases in the Middle East and are responsible for the lion's share of infant mortality, morbidity, and handicaps in Arab countries (*10*). Genetic disorders such as hemoglobinopathy, glucose-6-phosphate dehydrogenase deficiency, autosomal recessive syndromes, and several metabolic disorders have a presence throughout the Middle East (*10*). Patients and their advocates have pushed for awareness of hypoparathyroidism (lack of parathyroid hormone) (*11*) and beta thalassemia (a blood disorder that reduces the production of hemoglobin) (*12*). Other genetic disorders, *e.g.,* glutaric aciduria type I (an organic acid disorder where individuals cannot metabolize the amino acids lysine, hydroxylysine, and tryptophan), may be more specific to certain countries and subpopulations (in this case, Israel) (*13*).

Yet other rare diseases may not have a genetic cause but rather result from viral or bacterial infections or allergies. Behcet's disease, characterized by genital uclers, skin lesions, and uveitis, though very rare in the United States, is more common in the Middle East and

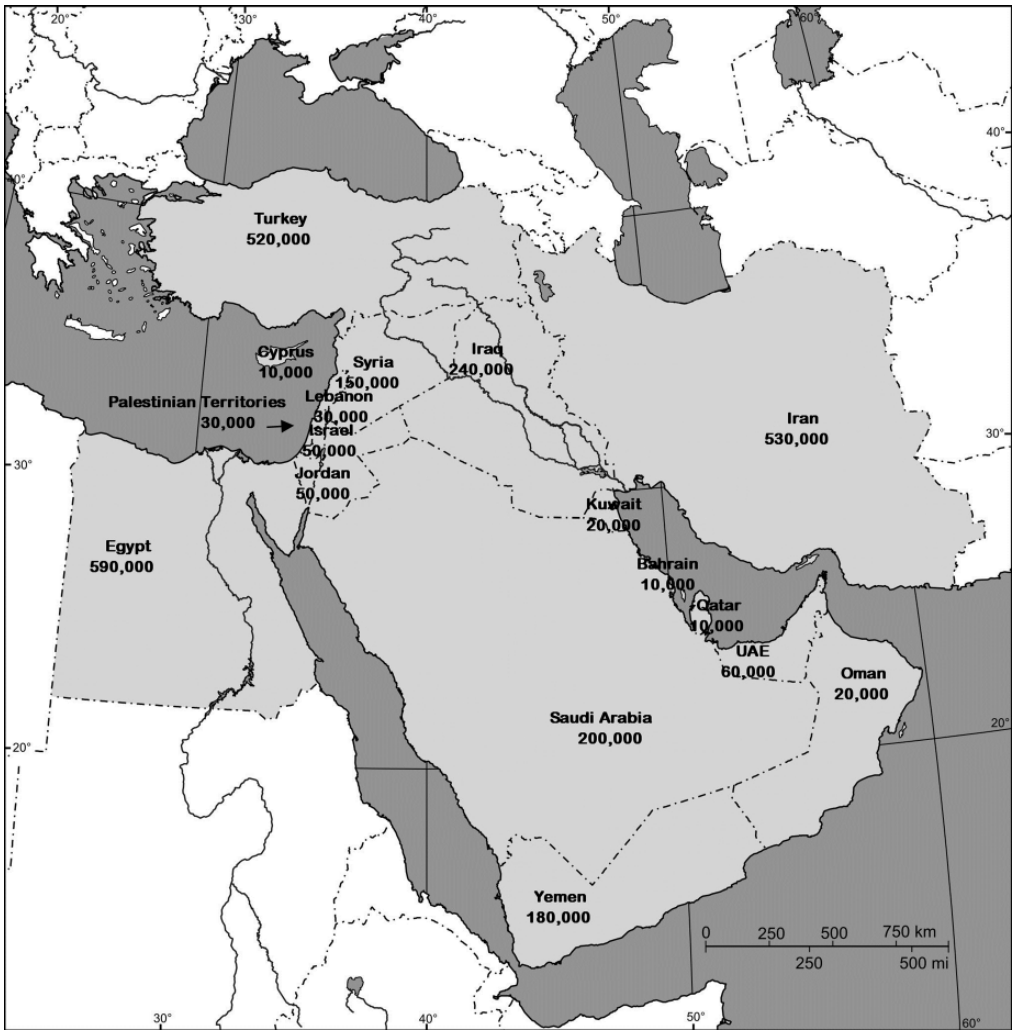


Figure 1. Estimated number of individuals with a rare disease in the Middle East (estimates obtained by multiplying United Nations' 2012 population estimates by 0.7% and rounding to the nearest 10,000). Source: Middle East outline map available at *http://www.zonu.com/fullsize-en/2009-11-17-11130/Middle-East-outline-map.html*. Accessed on September 21, 2012.

Asia, suggesting a tropical-area cause (*14*). The lichen planus pigmentosus presents with hyperpigmented, dark-brown macules in sun-exposed areas of the body and is more common in the Middle East than in Europe. It may be caused by a viral infection or topical agent (*15*). Leishmaniasis, a sand-fly-transmitted disease, leads to symptoms ranging from cutaneous lesions to fatal visceral disease. It has been designated as one of the most neglected tropical diseases by the World Health Organization (*16*). Pemphigus is the general designation for a group of autoimmune skin diseases that cause ulceration and crusting of the skin (*17*). It is present in people of Middle Eastern or Jewish descent (*18*).

3. Diagnosis of rare diseases in the Middle East

Orphan diseases are so rare that a physician will not observe a case often. In order to diagnose accurately a rare disease, doctors rely on the published literature and rare-disease registries, which vary considerably in volume and availability across rare diseases. Misdiagnosis or delayed diagnosis is very risky for rare-disease patients. For example, delaying the treatment for infantile-onset Pompe disease until the patient is 6 months old is already too late (*19*). Another problem with diagnosis is that there is no special coding system for rare diseases. The International-Classification-of-Diseases system that is used in most countries is not suitable for rare diseases. The absence of a universally recognized coding system is an obstacle for reliable registration of patients in national or international databases (*20*).

On top of the worldwide difficulty in diagnosing rare diseases, Middle Eastern countries generally have a shortage of trained medical professionals, partly due to the lack of medical schools in some of the countries and partly due to limited training in certain medical specialties including diagnostic medicine. The growing demand for physicians and other medical workers is currently being met in the Middle East partly by expatriates from the West, as well as from the Indian subcontinent and the Philippines, all of whom are unlikely to be trained in Arab rare diseases (*8*). However, Bahrain employs a relatively high proportion of nationals in healthcare; Dubai has attracted Harvard Medical School to Dubai Healthcare City where nationals will be trained; and Qatar is building a specialty teaching hospital run in association with Weill Cornell Medical College. Saudi Arabia is sending nationals abroad for training while it builds more teaching hospitals with the help of private investment (*8*). Basic training and continuing professional development are needed to ensure that all doctors have the ability to detect a rare disease, especially one more likely to occur in the Arab countries.

Meanwhile, efforts are being organized to keep track of patients with rare diseases. The Centre for Arab Genomic Studies (CAGS) launched a pilot project to construct the Catalogue of Transmission Genetics in Arabs (CTGA) database. This database helps Middle Eastern governments educate the medical community and raise public awareness in at-risk populations (*21*). In addition, Kuwait University established the Molecular Genetics Diagnostic Service Division, within the Faculty of Medicine, Department of Pathology, which focuses on delivering state-of-the-art genetic analysis for the Kuwaiti population. This service includes autozygosity (homozygosity in which two alleles are identical by descent) mapping in families with consanguineous marriages (*22*). Pre-marital genetic screening is offered in a number of countries, including Saudi Arabia, Bahrain, the UAE, and Jordan (*10*). A comprehensive program for thalassaemia screening and genetic counseling was started in Iran in 1996 (*10*).

4. Availability of orphan drugs in the Middle East

Orphan drugs are very expensive. Insurers in the United States have traditionally covered these therapies because only a small number of patients have needed them. However, as more new products are launched, payers will become more and more sensitive to cost, potentially affecting utilization (*23*). Some countries in the Middle East with per-capita income approaching or exceeding that of the United States should be able to pay for the drugs through public or private health insurance, though they will eventually face the same problems that United States payers are facing. Patients in other lower-income Arab countries, however, may have to rely on charitable organizations. Several of the countries in the region, including Qatar, Saudia Arabia, and Bahrain, are committed to increasing the role of the private sector in the public-private mix. The first private hospital in Qatar opened in 1999 (*8*). In 2008, expatriate health insurance became mandatory in Saudi Arabia for firms employing foreigners (*8*).

Orphan Europe, established in 1990, is a pharmaceutical company that develops and distributes orphan drugs. Today, the company provides 9 orphan products to patients all over the world. One of its products, Cystadane (betaine anhydrous), has marketing authorization in the United States, Canada, Australia, and Israel (*24*). It treats homocystinuria (an inherited rare condition where the body is unable to metabolize certain amino acids properly). This condition seems to be more common in some countries, including Qatar, where it is estimated that 1 in 1,800 people is affected (*25*). Orphan Europe has an office in Dubai Healthcare City. This move should help to increase awareness of rare diseases and orphan drugs in the Middle East.

Taiba is a leading regional specialty healthcare company, focused on marketing and distribution of pharmaceutical products for rare diseases. It acquires

and licenses innovative orphan drugs by building a strong network with international partners (*26*). The head office is in Muscat, Oman, and there is a regional office in Dubai. Indeed, leading pharmaceutical companies are turning to Taiba to help grow their business. For example, Dyax Corporation has given exclusive distribution rights to Taiba for the distribution of Kalbitor (ecallantide) in the Middle East (*27*). Kalbitor is used for the treatment of hereditary angioedema (a genetic defect that results in episodes of swelling in various parts of the body).

5. Development of orphan drugs in the Middle East

Orphan drug development in the United States took off after the passage of the Orphan Drug Act (ODA) of 1983. Under the ODA, drugs, vaccines, and diagnostic agents would qualify for orphan status if they were intended to treat a disease affecting fewer than 200,000 American citizens (*28*). Orphan drug designation means that the drug-company sponsor qualifies for certain benefits, including 7-year market exclusivity, tax incentives, and grants for drug development (*28*). Furthermore, the Food and Drug Administration (FDA) has been expediting the marketing approval for many orphan drugs (*29*). In 1982, only 34 drugs were marketed in the United States to treat orphan diseases (*30*). From the passage of the ODA until May 2010, the FDA approved 353 orphan drugs and granted orphan designations to 2,116 compounds (*31*). The European Union enacted legislation similar to the ODA in 1999. In Europe, an orphan designation is granted only if "… it is unlikely that the revenue after marketing of the medicinal product would cover the investment in its development" (*32*). Because of the nature of orphan drugs, no country can expect private-sector firms to embrace their development without public-sector legislation offering companies a potential return on their investment. It is simply much more profitable for them to develop drugs that can benefit large numbers of people globally.

Short of encouraging the growth of a domestic pharmaceutical industry, of course, attracting foreign investment is another option for countries in the Middle East. Indeed, the Dubai Biotechnology & Research Park (DuBiotech) provides an environment for life sciences companies to set up operations in the Middle East and to collaborate in productive partnerships, potentially with local firms (*33*). Pharmaceutical companies in DuBiotech include Pfizer, Genzyme, Merck-Serono, Amgen, Maquet, and Fimenich. Pharmax Pharmaceuticals, a "home-grown" pharmaceutical company in the United Arab Emirates that manufactures oral solid dosage products including tablets and capsules, will develop a 90,000-square-foot manufacturing facility, becoming the first pharmaceutical production unit at DuBiotech (*33*). Eventually, a successful development effort will require education and training in pharmaceutical sciences at Middle Eastern or foreign universities. The size of the global orphan-drugs market was about $84.9 billion in 2009, and the market is expected to reach $112.1 billion by 2014 (*34*). There will be profit-making opportunities for firms from all over the world, including from the Middle East, provided that some governmental incentives are in place.

Not all orphan drug development has to be executed by private pharmaceutical companies, with incentives from the government, although this has been the development model in the West. In the Middle East, public-private partnerships (PPPs) are public-health-driven, not-for-profit organizations that encourage pharmaceutical companies to develop new orphan drugs for rare diseases in partnership with them. Even large, multinational pharmaceutical companies may find it in their long-term interest to participate in the neglected-disease market provided that they can partner with experts in distribution and patient needs in developing countries. As of 2004, eight neglected-disease projects (Artemotil, Paluther, Coartem tablets pediatric label extension, Lapdap, Biltricide, Impavido, Ornidyl, and Mectizan) had been conducted in public-industry collaboration (*35*). One of the resulting products that had a major impact in the Middle East is Biltricide (praziquantel), which helps to control schistosomiasis (a parasitic disease). The PPPs model may, in at least some situations, be able to deliver better health care, both more efficiently and less expensively, than either the (primarily Western-based) private drug companies or the not-for-profit or public sector acting alone (*35*).

6. Conclusion

As healthcare improvement rises in priority in Middle Eastern countries, as a natural consequence of economic development, a focus on rare diseases and orphan drugs is to be expected. Although some diseases, cultural environment, and health-system features are uniquely Arab, ideas for government legislation and an optimal public-sector-private-sector mix in orphan-drug distribution and development can come from other countries that are currently paving the way. Ultimately, the rare-disease-and-orphan-drug problem is global. International discourse and cooperation should be at the top of every country's list of relevant policies.

Acknowledgements

The authors would like to thank Elizabeth Kunk, RN, BSN, for her help on an earlier draft of this article.

References

1. Aronson JK. Rare diseases and orphan drugs. Br J Clin

Pharmacol. 2006; 61:243-245.
2. Sharma A, Jacob A, Tandon M, Kumar D. Orphan drug: Development trends and strategies. J Pharm Bioallied Sci. 2010; 2:290-299.
3. Smith A. Cashing in on 'orphans'. CNN Money 2007. *http://money.cnn.com/2007/03/13/news/companies/genzyme/index.htm* (accessed September 21, 2012).
4. U.S. Food and Drug Administration. FDA approves first treatment for Pompe disease. *http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2006/ucm108645.htm* (accessed September 21, 2012).
5. U.S. Food and Drug Administration. FDA Approves new treatment for late-onset Pompe disease. *http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2010/ucm213282.htm* (accessed September 21, 2012).
6. Werber Y. Lysosomal storage diseases market. Nat Rev Drug Discov. 2004; 3:9-10.
7. Wang JB, Guo JJ, Yang L, Zhang YD, Sun ZQ, Zhang YJ. Rare diseases and legislation in China. Lancet. 2010; 375:708-709.
8. Grant Thornton International. Transforming the Middle East's healthcare model. Healthcare Guide 2009. *http://www.grantthornton.com/staticfiles/GTCom/Grant%20Thornton%20Thinking/International/GTI_ME_Healthcare_Report_2009.pdf* (accessed September 21, 2012).
9. United Nations, Department of Economic and Social Affairs. Annual population 2011-2100 both sexes. *http://esa.un.org/unpd/wpp/Excel-Data/population.htm* (accessed September 21, 2012).
10. Al-Gazali L, Hamamy H, Al-Arrayad S. Genetic disorders in the Arab world. British Medical Journal. 2006; 333:831-834.
11. Torr R. Drive to fight rare disease. The Voice of Bahrain. Gulf Daily News 2008. *http://gulf-daily-news.com/NewsDetails.aspx?storyid=224196* (accessed September 21, 2012).
12. Al Hajeri A, Al Arrayed S. Public awareness of beta thalassemia in Bahrain. Bahrain Medical Bulletin. 2012; 34:7 pages. *http://www.bahrainmedicalbulletin.com/march_2012/Public_Awareness.pdf* (accessed September 21, 2012).
13. Anikster Y, Shaag A, Joseph A, Mandel H, Ben-Zeev B, Christensen E, Elpeleg ON. Glutaric aciduria type I in the Arab and Jewish communities in Israel. Am J Hum Genet. 1996; 59:1012-1018.
14. American Behcet's Disease Association. Most common symptoms and signs of Behcet's disease. *http://www.behcets.com/site/pp.asp?c=bhJIJSOCJrH&b=260548* (accessed September 21, 2012).
15. French National Institute of Health and Medical Research. Orphanet. *http://www.orpha.net/consor/cgi-bin/index.php?lng=EN* (accessed September 21, 2012).
16. McDowell MA, Rafati S, Ramalho-Ortigao M, Ben Salah A. Leishmaniasis: Middle East and North Africa research and development priorities. PLoS Negl Trop Dis. 2011; 5: e1219.
17. National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS). Questions and answers about Pemphigus. *http://www.niams.nih.gov/Health_Info/Pemphigus/default.asp* (accessed September 21, 2012).
18. Pemphigus. MayoClinic.com. 2008. *http://www.mayoclinic.com/print/pemphigus/DS00749/METHOD=print&DSECTION=all* (accessed September 21, 2012).
19. Tang W, Makuuchi M. Intractable and rare diseases research. Intractable & Rare Diseases Research. 2012; 1:1-2.
20. Song P, Gao J, Inagaki Y, Kokudo N, Tang W. Rare diseases, orphan drugs, and their regulation in Asia: Current status and future perspectives. Intractable & Rare Diseases Research. 2012; 1:3-9.
21. Tadmouri GO, Al Ali MT, Al-Haj Ali S, Al Khaja N. CTGA: The database for genetic disorders in Arab populations. Nucleic Acids Res. 2006; 34 (Database issue):D602-D606.
22. Patrinos GP, Al Aama J, Al Aqeel A, Al Mulla F, Borg J, Devereux A, Felice AE, Macrae F, Marafie MJ, Petersen MB, Qi M, Ramesar RS, Zlotogora J, Cotton R. Recommendations for genetic variation data capture in developing countries to ensure a comprehensive worldwide data collection. Hum Mutat. 2011; 32:2-9.
23. Hyde R, Dobrovolny D. Orphan drug pricing and payer management in the United States: Are we approaching the tipping point? American Health & Drug Benefits. 2010; 3:15-23.
24. Recordati Group. Orphan Europe. *http://www.orphan-europe.com/* (accessed September 21, 2012).
25. National Library of Medicine. Genetics Home Reference. *http://ghr.nlm.nih.gov/* (accessed September 21, 2012).
26. Taiba Managing Medicines in Middle-East. *http://www.taibame.com/about.php* (accessed September 21, 2012).
27. Dyax Corporation. Dyax announces partnership with Taiba for distribution of Kalbitor® (ecallantide) in the Middle East. *http://investor.dyax.com/releasedetail.cfm?ReleaseID=680023* (accessed September 21, 2012).
28. Villarreal MA. Orphan drug act: Background and proposed legislation in the 107th Congress. CRS Report for Congress. *http://www.policyarchive.org/handle/10207/bitstreams/3490.pdf* (accessed September 21, 2012).
29. Burton TM. Many 'orphan drugs' get expedited review by FDA. Wall Street Journal. *http://online.wsj.com/article/SB10001424052970204450804576623452161666840.html* (accessed September 21, 2012).
30. Rohde DD. The Orphan Drug Act: An engine of innovation? At what cost? Food Drug Law J. 2000; 55:125-144.
31. Armstrong W. Pharma's orphans. Pharmaceutical Executive. *http://www.pharmexec.com/pharmexec/article/articleDetail.jsp?id=670568&sk=&date=&pageID=4* (accessed September 21, 2012).
32. European Medicines Agency. Orphan designation. *http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000029.jsp&mid=WC0b01ac05800240ce* (accessed September 21, 2012).
33. Dubai Biotechnology & Research Park (DuBiotech). *http://www.dubiotech.ae/* (accessed September 21, 2012).
34 BCC Research. Global markets for orphan drugs. *http://www.bccresearch.com/report/PHM038C.html* (accessed September 21, 2012).
35. Moran M. A breakthrough in R&D for neglected diseases: New ways to get the drugs we need. PLoS Med. 2005; 2: e302.

(Received July 17, 2012; Revised September 25, 2012; Accepted September 26, 2012)

Review

DOI: 10.5582/irdr.2012.v1.4.144

Peripheral stimulation in treating Parkinson's disease: Is it a realistic idea or a romantic whimsicality?

Tetsuya Asakawa[1,*], Huan Fang[2], Zhen Hong[3], Kenji Sugiyama[1], Takao Nozaki[1], Hiroki Namba[1,*]

[1] *Department of Neurosurgery, Hamamatsu University School of Medicine, Hamamatsu, Japan;*
[2] *Department of Pharmacy, Jinshan Hospital of Fudan University, Shanghai, China;*
[3] *Department of Neurology, Huashan Hospital of Fudan University, Shanghai, China.*

Summary **Parkinson's disease (PD) is a common, however, intractable neurodegenerative disorder in the aging population. Levodopa (L-dopa) administration is regarded as the most effective strategy in treating PD with prominent motor side-effects after undergoing long-term treatment. Surgical therapies such as deep brain stimulation (DBS) show certain efficacy, yet there are several limitations in adopting such surgical procedures. Therefore, performing electrical stimulation out of the brain, namely peripheral stimulation for PD has been a dream of many clinicians. Recently, the efficacy of dorsal column stimulation was verified in animal PD models; on the other hand, tons of acupunctural studies from East Asia claim good efficacy in treating PD both in bench and clinical studies. This review will introduce the progress of peripheral stimulation for PD, and will discuss the potential mechanisms involved in these strategies.**

Keywords: Parkinson's disease, deep brain stimulation, peripheral stimulation, dorsal column, acupuncture, somatosensory system

1. Introduction

Parkinson's disease (PD), first reported by James Parkinson (1817), is a progressive neurodegenerative disorder which is common in the elder population with an unclear pathogenesis (*1*). The pathophysiological hallmark is the progressive degeneration of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc), and the main symptoms are static tremor, rigidity, bradykinesia, gait dysfunction and postural instability (*2,3*). The detailed pathogenesis remains unclear. It is believed that PD is a comprehensive result of genetic factors and environmental toxins (Figure 1) (*4*).

There is no perfect strategy in treating PD. Medical therapy is still playing the most important role for PD. Currently, levodopa (L-dopa) is certainly the best medicine for idiopathic PD. Other medicines such as dopamine receptor agonists, monoamine oxidase-B (MAO-B) inhibitors, amantadine and anticholinergic medications are also used as adjuvant drugs for L-dopa with functions of reducing the dose of L-dopa, or prolonging L-dopa's effective time, or releasing the side-effects of L-dopa (*4*). Traditional surgical processes include old surgical ablation (pallidotomy or thalamotomy) and newer high-frequency deep brain stimulation (DBS) of certain structures such as the subthalamic nucleus (STN). STN-DBS has been proved as an effective therapy both in clinical reports (*5*) and animal studies (*6-8*). The mechanisms of such traditional surgical therapies are unclear. It is believed that the surgical ablation or STN-DBS breaks the motor controlling circuits concerning the basal ganglia, while DBS corrects the overactive state of STN in the PD state (*9-11*).

If we define the medical and surgical treatments

**Address correspondence to:*
Dr. Tetsuya Asakawa, Department of Neurosurgery, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan. E-mail: asakawat1971@gmail.com

Dr. Hiroki Namba, Department of Neurosurgery, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan. E-mail: hnamba@hama-med.ac.jp

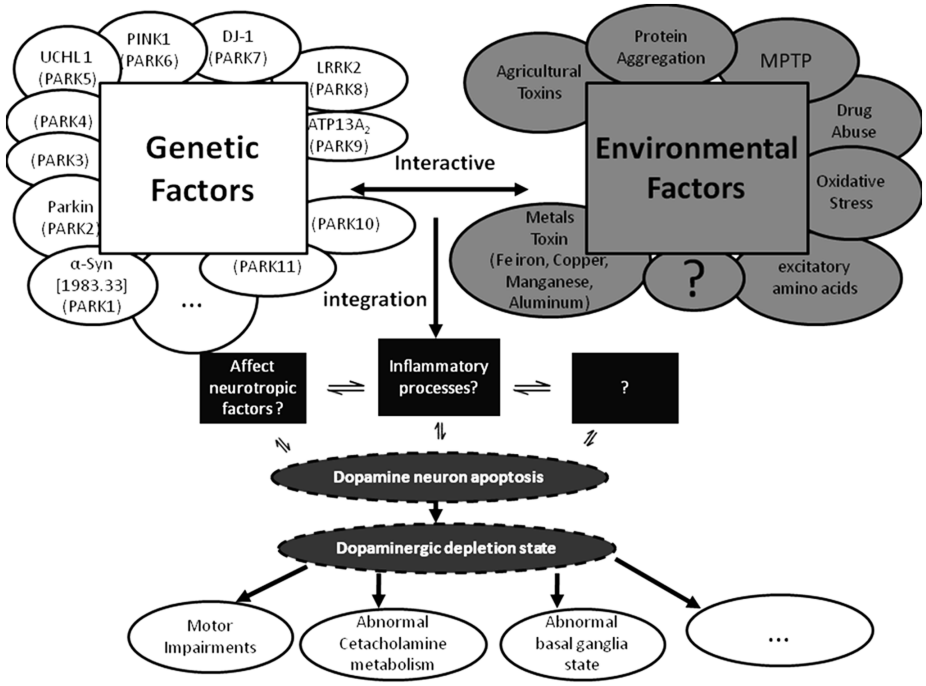


Figure 1. The possible mechanisms involved in PD pathogenesis (*Asakawa and Xia, 2012*).

as "classic treatments", the next generation treatments for PD should include gene therapy and stem cell transplantation. At present, the main solutions of gene therapy include two directions: one is to improve the cerebral neurotrophic factors, including the brain derived neurotrophic factor (BDNF) (*12-14*) and glial cell line-derived neurotrophic factor (GDNF) (*15,16*). However, some clinical trials regarding GDNF produced incompatible results, and thus the efficacy of improving cerebral GDNF needs more evidence (*17-19*). Another direction of gene therapy is to enhance GABA expression of STN by transfer of the glutamic acid decarboxylase (*GAD*) gene using adeno-associated virus (AAV) (*20*). It is hopeful that the method will be acceptable as a new treatment. The most challenging/dramatic next generation therapy is stem cell transplantation. The development of induced pluripotent stem cells (iPSCs) resolved the derivation of stem cells, and has allowed using stem cells to "make" DA neurons which is a promising strategy for DA. However, technical problems such as a low success rate in making DA neurons *in vivo* and a high cancer rate hold back the clinical application of stem cells.

2. The limitations of the classic therapies currently

All the classic treatments, including medical and surgical, are symptomatic therapies, which contribute little to stop/ameliorate neuron degeneration progression. Such symptomatic therapies have many weaknesses, and are far from satisfactory therapies. In this regard, PD is always thought of as an intractable disease.

L-dopa administration is regarded as the most effective therapy currently. Most of the patients experience a dramatic improvement during the early stage of treatment. Unfortunately, with the progress of PD, the dose of L-dopa has to be enhanced to achieve the same efficacy (wearing off sign). At the advanced stage, the efficacy becomes weaker, and some motor side-effects appear. Such motor side-effects of L-dopa always emerge along with the motor symptoms, which make the patients always suffer from severe motor dysfunction (*4*).

As to the surgical processes, several limitations are reported in the previous studies: *i*) The mechanisms of surgical treatments remain unclear, which will influence clinical practice using such therapies. For instance, parameter selection is a tough problem faced by the clinicians and patients undergoing DBS. Albeit the high frequency, experiential pulse (about 60 μs) is accepted by most of the researchers (*11,21,22*), the stimulation current intensity is a difficult problem. Recently, several animal studies revealed that the best parameters to ameliorate contrasting symptoms are quite different (*7,8*). The parameter selection is individualized and experience-based in different patients. Moreover, the problem of the battery life of the stimulator embedded under the skin can not be ignored. This problem usually forces the patients to make a tough decision: either to undergo another surgical operation to change the battery, or adopt a palliative pattern by reducing the stimulation current to save the battery (*4*). *ii*) DBS is an invasive therapy with a high surgical risk, and the long-term efficacy is also uncertain. Although several

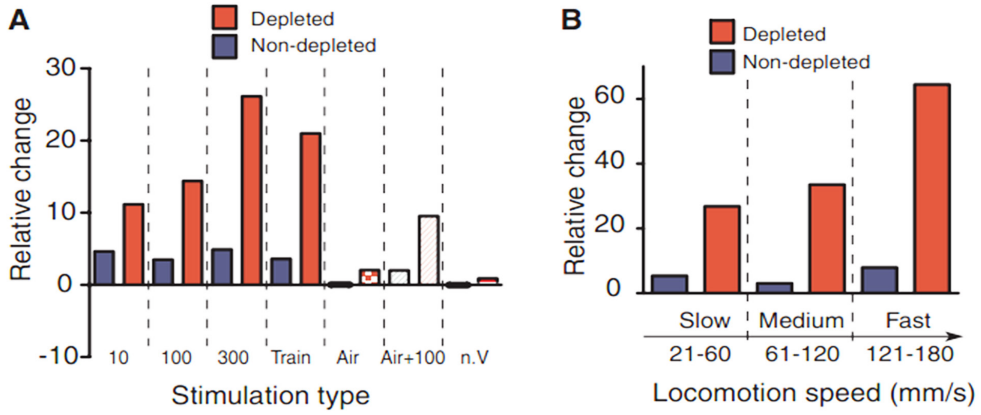


Figure 2. DCS improved the locomotion in DA-depleted mice (*Fuentes et al., 2009*). (A), Stimulation of dorsal columns enhanced the locomotion in mouse PD models, while 300 Hz stimulation achieved the best efficacy; **(B)**, DCS significantly ameliorates the fraction of faster movement components in mouse PD models.

clinical studies claimed a good efficacy for DBS after long-term observation (*23,24*), the significant adverse events reported and the battery life of the stimulator can not be ignored. *iii*) DBS is an expensive process, which can not be popularized in some developing countries without a good health insurance system.

There are so many limitations of the classic medical and surgical therapies currently, and there is still a long way to go to apply the next generation treatments clinically. To explore a safe (less invasive), low-cost, however effective therapy is a dream of all PD clinicians. In this regard, peripheral stimulation is taken into account.

3. Overview of peripheral stimulation

DBS is an invasion strategy with high surgical risk for PD. If the electrical stimulation can be performed out of the brain, the invasiveness, risk and cost will be profoundly reduced, while the operation process can be dramatically simplified. We defined such performing electrical stimulation at structures out of the brain, such as spinal cord, peripheral nerve, muscle, skin, *etc.*, as peripheral stimulation. Currently, mainly two sorts of peripheral stimulations are reported, namely spinal cord electrical stimulation (*25*) and acupuncture (*4*).

Fuentes in 2009 first reported that epidural electrical stimulation of dorsal columns in the spinal cord improves motor impairments in both rat and mouse PD models (*25*). They used acute pharmacologically induced DA-depleted mice and chronic 6-hydroxydopamine (6-OHDA)-lesioned rats. Dorsal column stimulation (DCS) was performed and evaluated in these models. They found 300 Hz stimulation dramatically enhanced the amount of locomotion during the stimulation period compared to the control (Figure 2A). DCS also contributed to the alleviation of bradykinesia since fast-movement components are significantly ameliorated (Figure 2B). They also found that DCS affected the firing patterns of individual neurons. When performing DCS in combination with L-dopa administration, they found DCS achieved a 4/5 dose reduction of L-dopa to reach the same efficacy (Figure 3A). Such results were repeated in 6-OHDA-lesioned rat models (Figures 3B and 3C). However, the subsequent clinical study produced incompatible results (*26,27*). High-frequency epidural cervical spinal cord stimulation was performed for two PD patients using different frequencies and current intensities. Unfortunately, they did not find any significant difference (Table 1). DCS stimulation is a total new approach for PD, and the mechanisms are unknown (we will discuss it in the next section). More bench and clinical studies should be involved since the dorsal column may be a potential target for peripheral stimulation.

Acupuncture is another method reported to claim "good efficacy" for treatment of PD by stimulation out of the brain (*4*). Acupuncture is an alternative therapy which achieves improvement of certain diseases by stimulation of acupoints at the body surface, based on the theories of traditional Chinese medicine (TCM). Acupuncture is popular in east Asia. Tons of papers published in China and Korea claimed good efficacy of acupuncture in treating PD. However, most of these studies are poorly designed, and therefore we can not get rigorous evidence to evaluate the efficacy of acupuncture. Lee in 2008 investigated the acupuncture studies and found only 11 of the 103 studies reached a level of randomized controlled trials (RCTs) with subjective outcome measures, while only 1 study described a double-blind method. This rigorously designed study by Cristian *et al*. could not find any efficacy for acupuncture in treating PD (*28,29*). Lam in 2008 evaluated acupuncture studies available in the database, and

found only 10 of 784 can be attributed to RCTs, however, there are still flaws in the experimental design in these 10 studies (*30*). Asakawa in 2012 reviewed 2,354 original studies using acupuncture to treat PD, and could not find even one paper providing believable evidence to prove acupuncture's efficacy (Table 2). He summarized the main flaws involved in the acupuncture studies and aroused large, well-designed and multicenter clinical trials to evaluate the efficacy and safety of acupuncture (*4,31*).

Albeit peripheral stimulation is an attractive and hopeful approach for PD, unfortunately at present there is no powerful evidence to prove efficacy clinically, either in spinal cord stimulation, or in acupuncture. More studies should be engaged in these two directions since peripheral stimulation is a good clue for developing low-invasion PD treatment.

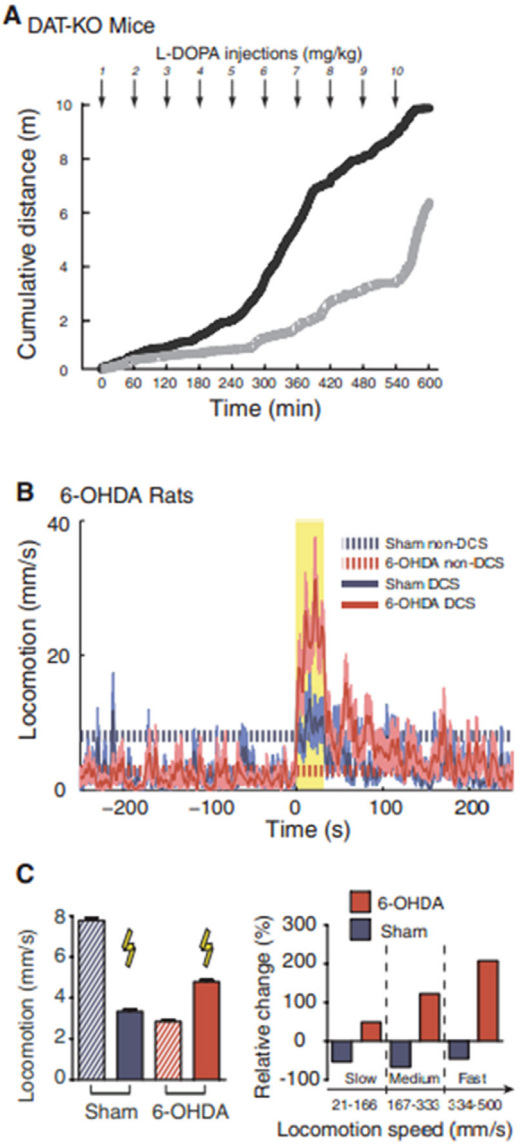


Figure 3. DCS improved locomotion in severely DA-depleted mice and in chronically lesioned rats (*Fuentes et al., 2009*). (A), DCS established better efficacy in the group undergoing DCS in combination with successive L-dopa injections (black) than the group only receiving L-dopa (gray); **(B)**, DCS (yellow shaded area) caused significant improvements of locomotion in 6-OHDA-lesioned rats (shaded area around trace is SEM); **(C)**, (Left) DCS specifically improved locomotion in 6-OHDA-lesioned rats, (Right) Faster movement components of locomotion were also ameliorated by DCS in the 6-OHDA-lesioned rats.

4. The potential mechanisms involved in peripheral stimulation

Until now, we still do not know whether peripheral stimulation can be employed as a candidate new treatment for PD. If the potential mechanisms can be clarified, it will be helpful to develop effective peripheral stimulation.

4.1. *The somatosensory system, a bridge between peripheral stimulation and the dopaminergic system?*

The essence of this problem is that if stimulation of peripheral structures can affect the cerebral dopaminergic system. Several reports revealed that peripheral electrical stimulation is able to affect cerebral DA release. As far back as 1977, Nieoullon found electrical stimulation of the cats' forepaw resulted in DA release which was reduced in the ipsilateral substantial nigra and enhanced in the caudate nucleus (*32*). Subsequently, several studies found stimulation of the somatosensory system affects the dopaminergic system, which is related to motor function (Figure 4) (*33-35*). These findings all indicated a close connection between the somatosensory system and DA system. However, the anatomical structure, distribution and circuits of the somatosensory system are poorly understood. Inoue's 2004 paper deduced the possible anatomical pathways which exist between the mesencephalic DA-ergic nuclei and the sensory system causing the observed modulation of DA release in the basal ganglia. One important plausible connection is from the sensory areas in the contralateral neocortex which projects back into the ipsilateral striatum, and then activates ipsilateral DA release from mesencephalic DA-ergic nuclei.

Table 1. DCS did not conduct any significant amelioration in two PD patients (*Thevathasan et al., 2010*)

	Motor UPDRS (score/104)	Timed10-meter walk (s)	Timed hand-arm movements (n/30s)	Timed lower limb tapping (n/30s)
baseline (off stimulation)	37.8 (11.5)	5.5 (1.2)	30.3 (15.9)	54.2 (21.9)
subthreshold stimulation	35.4 (12.5)	5.4 (0.4)	32.7 (18.0)	54.2 (22.8)
suprathreshold stimulation	37.3 (10.5)	5.6 (1.0)	31.2 (16.3)	52.0 (24.7)
friedman (*p* value)	0.44	0.72	0.32	0.85

Table 2. The common flaws in the experimental design of the acupunctural studies. Modified from the chapter of (*Asakawa and Xia, 2012*)

Weaknesses in the experimental design	Comments	Solutions
The undefined sample size	Sample size is the most crucial aspect of formulating an experimental design.	1. Estimated according to the statistical principles (*Asakawa and Xia, 2012*). 2. The method for establishing the sample size should be clearly put down.
Lack of "blinding"	Basically, double-blinding is needed; however, single blinding with objective indices is acceptable.	Methods of Allocation concealment should be included in the paper.
Insufficient randomization or pseudorandom	Only a small number of these studies used a robust randomization procedure while many of them used insufficient randomization, even pseudo-random in some cases.	1. Simple randomization. 2. Block randomization. 3. Stratified randomization. 4. Covariate adaptive randomization.
Inappropriate control group setting	There are several flaws in using the popular protocol like C + A *vs*. C (C = classical therapy; A = acupuncture).	1. A protocol of A + C *vs* C + P, (P = placebo acupuncture) is recommended. 2. The methodology of setting placebo acupuncture.
Lack of objective evaluation standard	1. Lack of an objective standard for acupoint-selection. 2. No standardized method of performing manual acupuncture. 3. The evaluation criteria are also subjective.	The ideas of evidence-based medicine should be set up. Change the traditional experience-based ideas.
Weakness in statistical analysis	1. Fail to describe the data analysis methods. 2. List only raw data in tables , do not use any figure with statistical information.	Consult a statistical expert. Using statistical figures.
Neglect in recording adverse events and withdrawals	Without adequate reporting of adverse events and withdrawals, the results from a clinical trial can not be accepted.	Adverse events and withdrawals should be recorded clearly.

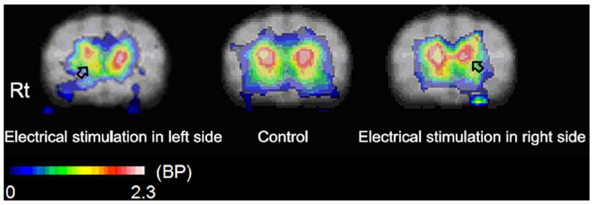


Figure 4. DA-release marked by striatal D2-like receptor activity was affected by forepaw electrical stimulation (*Inoue et al., 2004*). (Left), The electrical stimulation in the left paw decreased the BP value in the right basal ganglia; **(Center)**, A parametric image of BP without stimulation. (Inoue *et al.*, 2004); **(Right)**, The electrical stimulation in the right paw decreased the BP value in the left basal ganglia. The BP value is shown in the color bar in coregistered MRI. Rt: The right side of the cat brain.

Another possible anatomical connection could be from the projecting fibers between the nuclei intralaminares thalami and the SNpc through the striatum on the contralateral side. Furthermore, there is another potential pathway between the ventral tegmental area and the mesencephalic central gray area, which is innervated collaterally by the spinothalamic tract (*4,33)*.

The mysterious somatosensory pathways may play a role in the connection between peripheral stimulation and the DA system. We hypothesize that the dorsal column and the effective acupoints (if the efficacy can be strictly verified) should be the "stations" of the somatosensory pathways. The motor functions related to the dopaminergic system are then affected through the somatosensory pathways when the "stations" are undergoing electrical stimulation.

4.2. *DCS unlocks the basal ganglia-cortical circuits?*

Besides the dopaminergic system, another important possibility is that DCS (or effective acupuncture) activates the locked basal ganglia-cortical circuits in the PD state. In a later paper to explain the mechanisms of DCS, Fuentes pointed out that the improvement of motor function might be the result of basal ganglia-cortical circuits being unlocked and conducted by synchronous stimulation of a number of tactile afferent fibers terminating in the dorsal column nuclei and ascending through the lemniscal pathway to cortical areas through the thalamus, and the thalamic nuclei most directly activated by DCS differ from those primarily affected by STN-/GPi-DBS. In addition, activating the pedunculopontine nucleus (PPN) through some ascending and descending anatomical tracers from the cervical and thoracic spinal cord dorsal horns projects directly to PPN and may play a role in the mechanisms of DCS (*36*). It has been well investigated that activation of PPN contributes to improvement of the initiation of movement through a descending drive to locomotor circuits directly; and activation/desynchronization of the motor cortex along with certain structures within the basal ganglia through ascending thalamocortical pathways indirectly (*36-38*).

No matter what the mechanisms are concerning the dopaminergic system, or concerning the basal ganglia circuits, achieving deeper understanding of the potential anatomical connections is crucial. It may be a key to uncover the secrets of peripheral stimulation.

5. Conclusion

Although only one rigorously designed bench study (*25*) verified the efficacy of peripheral stimulation in treating PD, we can expect the possibility of treating PD by stimulation outside of the brain. More bench and clinical studies should be designed and verified for peripheral stimulation. Certainly the efficacy of acupuncture should be also strictly verified. The "effective" acupoints may be employed in affording hints of stimulating targets or finding the "stations" of unknown anatomical connections.

Acknowledgements

TA was supported by grants from the Japan Society for the Promotion of Science (Grant-in-Aid for Young Scientists, Type B, No. 20791025 and Grant-in-Aid for Scientific Research C, General, No. 24592157).

References

1. Parkinson J. An essay on the shaking palsy. 1817. J Neuropsychiatry Clin Neurosci. 2002; 14:223-236; discussion 222.
2. Hirsch E, Graybiel AM, Agid YA. Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. Nature. 1988; 334:345-348.
3. Przedborski S. Pathogenesis of nigral cell death in Parkinson's disease. Parkinsonism Relat Disord. 2005; 11 (Suppl 1):S3-S7.
4. Asakawa T, Xia Y. Acupuncture Treatment For Parkinso's Disease, in Current Research in Acupuncture (Xia Y, Ding G, eds.). Springer, New York, USA, 2012; pp. 215-255.
5. Benabid AL, Koudsié A, Benazzouz A, Fraix V, Ashraf A, Le Bas JF, Chabardes S, Pollak P. Subthalamic stimulation for Parkinson's disease. Arch Med Res. 2000; 31:282-289.
6. Fang X, Sugiyama K, Akamine S, Namba H. Improvements in motor behavioral tests during deep brain stimulation of the subthalamic nucleus in rats with different degrees of unilateral parkinsonism. Brain Res. 2006; 1120:202-210.
7. Asakawa T, Sugiyama K, Akamine S, Yokoyama C, Shukuri M, Mizuma H, Tsukada H, Onoe H, Namba H. The food reaching test: A sensitive test of behavioral improvements by deep brain stimulation in MPTP-treated monkey. Neurosci Res. 2012; 74:122-128.
8. Fang X, Sugiyama K, Akamine S, Sun W, Namba H. The different performance among motor tasks during the increasing current intensity of deep brain stimulation of the subthalamic nucleus in rats with different degrees of the unilateral striatal lesion. Neurosci Lett. 2010; 480: 64-68.
9. Alexander GE, Crutcher MD, DeLong MR. Basal ganglia-thalamocortical circuits: Parallel substrates for motor, oculomotor, "prefrontal" and "limbic" functions. Prog Brain Res. 1990; 85:119-146.
10. Alexander GE, Crutcher MD, DeLong MR. Parallel organization of functionally segregated circuits linking basal ganglia and cortex. Annu Rev Neurosci. 1986; 9: 357-381.
11. Nambu A, Tokuno H, Takada M. Functional significance of the cortico-subthalamo-pallidal 'hyperdirect' pathway. Neurosci Res. 2002; 43:111-117.
12. Frim DM, Uhler TA, Galpern WR, Beal MF, Breakefield XO, Isacson O. Implanted fibroblasts genetically engineered to produce brain-derived neurotrophic factor prevent 1-methyl-4-phenylpyridinium toxicity to dopaminergic neurons in the rat. Proc Natl Acad Sci U S A. 1994; 91:5104-5108.
13. Liang XB, Liu XY, Li FQ, Luo Y, Lu J, Zhang WM, Wang XM, Han JS. Long-term high-frequency electro-acupuncture stimulation prevents neuronal degeneration and up-regulates BDNF mRNA in the substantia nigra and ventral tegmental area following medial forebrain bundle axotomy. Brain Res Mol Brain Res. 2002; 108:51-59.
14. Shults CW, Kimber T, Altar CA. BDNF attenuates the effects of intrastriatal injection of 6-hydroxydopamine. Neuroreport. 1995; 6:1109-1112.
15. Burke RE, Antonelli M, Sulzer D. Glial cell line-derived neurotrophic growth factor inhibits apoptotic death of postnatal substantia nigra dopamine neurons in primary culture. J Neurochem. 1998; 71:517-525.
16. Clarkson ED, Edwards-Prasad J, Freed CR, Prasad KN. Immortalized dopamine neurons: A model to study neurotoxicity and neuroprotection. Proc Soc Exp Biol Med. 1999; 222:157-163.
17. Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, Bunnage M, Brooks DJ, Svendsen CN, Heywood P. Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. Nat Med. 2003; 9:589-595.
18. Lang AE, Gill S, Patel NK, *et al*. Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. Ann Neurol. 2006; 59:459-466.
19. Slevin JT, Gerhardt GA, Smith CD, Gash DM, Kryscio R, Young B. Improvement of bilateral motor functions in patients with Parkinson disease through the unilateral intraputaminal infusion of glial cell line-derived neurotrophic factor. J Neurosurg. 2005; 102:216-222.
20. Kaplitt MG, Feigin A, Tang C, Fitzsimons HL, Mattis P, Lawlor PA, Bland RJ, Young D, Strybing K, Eidelberg D, During MJ. Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne *GAD* gene for Parkinson's disease: An open label, phase I trial. Lancet. 2007; 369:2097-2105.
21. Hashimoto T, Elder CM, Okun MS, Patrick SK, Vitek JL. Stimulation of the subthalamic nucleus changes the firing pattern of pallidal neurons. J Neurosci. 2003; 23:1916-1923.
22. Fang X, Sugiyama K, Akamine S, Namba H. The stepping test and its learning process in different degrees of unilateral striatal lesions by 6-hydroxydopamine in rats. Neurosci Res. 2006; 55:403-409.
23. Kleiner-Fisman G, Fisman DN, Sime E, Saint-Cyr JA, Lozano AM, Lang AE. Long-term follow up of bilateral deep brain stimulation of the subthalamic nucleus in patients with advanced Parkinson disease. J Neurosurg. 2003; 99:489-495.
24. Krack P, Batir A, Van Blercom N, Chabardes S, Fraix V, Ardouin C, Koudsie A, Limousin PD, Benazzouz A, LeBas JF, Benabid AL, Pollak P. Five-year follow-

up of bilateral stimulation of the subthalamic nucleus in advanced Parkinson's disease. N Engl J Med. 2003; 349: 1925-1934.
25. Fuentes R, Petersson P, Siesser WB, Caron MG, Nicolelis MA. Spinal cord stimulation restores locomotion in animal models of Parkinson's disease. Science. 2009; 323:1578-1582.
26. Nicolelis MA, Fuentes R, Petersson P, Thevathasan W, Brown P. Spinal cord stimulation failed to relieve akinesia or restore locomotion in Parkinson disease. Neurology. 2010; 75:1484-1485.
27. Thevathasan W, Mazzone P, Jha A, Djamshidian A, Dileone M, Di Lazzaro V, Brown P. Spinal cord stimulation failed to relieve akinesia or restore locomotion in Parkinson disease. Neurology. 2010; 74: 1325-1327.
28. Cristian A, Katz M, Cutrone E, Walker RH. Evaluation of acupuncture in the treatment of Parkinson's disease: A double-blind pilot study. Mov Disord. 2005; 20:1185-1188.
29. Lee MS, Shin BC, Kong JC, Ernst E. Effectiveness of acupuncture for Parkinson's disease: A systematic review. Mov Disord. 2008; 23:1505-1515.
30. Lam YC, Kum WF, Durairajan SS, Lu JH, Man SC, Xu M, Zhang XF, Huang XZ, Li M. Efficacy and safety of acupuncture for idiopathic Parkinson's disease: A systematic review. J Altern Complement Med. 2008; 14: 663-671.
31. Asakawa T, Xia Y. Future Research in Acupuncture – Better design and analysis for novel and valid findings, in Current Research in Acupuncture (Xia Y, Ding G, eds.). Springer, New York, USA, 2012; pp. 687-727.
32. Nieoullon A, Cheramy A, Glowinski J. Nigral and striatal dopamine release under sensory stimuli. Nature. 1977; 269:340-342.
33. Inoue M, Katsumi Y, Hayashi T, Mukai T, Ishizu K, Hashikawa K, Saji H, Fukuyama H. Sensory stimulation accelerates dopamine release in the basal ganglia. Brain Res. 2004; 1026:179-184.
34. Rothblat DS, Schneider JS. Response of caudate neurons to stimulation of intrinsic and peripheral afferents in normal, symptomatic, and recovered MPTP-treated cats. J Neurosc. 1993; 13:4372-4378.
35. Schultz W, Romo R. Responses of nigrostriatal dopamine neurons to high-intensity somatosensory stimulation in the anesthetized monkey. J Neurophysiol. 1987; 57: 201-217.
36. Fuentes R, Petersson P, Nicolelis MA. Restoration of locomotive function in Parkinson's disease by spinal cord stimulation: Mechanistic approach. Eur J Neurosci. 2010; 32:1100-1108.
37. Hikosaka O. Basal ganglia – possible role in motor coordination and learning. Curr Opin Neurobiol. 1991; 1: 638-643.
38. Jenkinson N, Nandi D, Muthusamy K, Ray NJ, Gregory R, Stein JF, Aziz TZ. Physiology, and pathophysiology of the pedunculopontine nucleus. Mov Disord. 2009; 24: 319-328.

(Received November 9, 2012; Accepted November 18, 2012)

Review

DOI: 110.5582/irdr.2012.v1.4.151

Classification and management of hepatolithiasis: A high-volume, single-center's experience

Xiaobin Feng[1], Shuguo Zheng[1], Feng Xia[1], Kuansheng Ma[1], Shuguang Wang[1], Ping Bie[1], Jiahong Dong[1,2,*]

[1] *Institute of Hepatobiliary Surgery, Southwest Hospital, Third Military Medical University, Chongqing, China;*
[2] *Institute of Hepatobiliary Surgery, Chinese PLA General Hospital, Beijing, China.*

Summary **Hepatolithiasis is endemic to East Asia, but immigration from the region means that this rare but emerging disease will pose a therapeutic challenge to doctors in the West as well. Curative management of hepatolithiasis is difficult since its etiology has not been fully elucidated. Hepatectomy is the best approach to treating hepatolithiasis. Here, we propose a novel classification of hepatolithiasis and describe features of each type. We then relate our experience with various forms of hepatectomy to treat different types of hepatolithiasis. Surgery should be indicated for all cases of hepatolithiasis. The proposed classification will help to determine surgical strategies. Better selection of which patients should undergo a hepatectomy will lead to better outcomes.**

Keywords: Hepatectomy, hepatolithiasis, classification

1. Introduction

Hepatolithiasis is endemic to East Asia, which includes China, South Korea, Japan, the Philippines, Vietnam, Thailand, Malaysia, and Indonesia, and its prevalence there can range as high as 30-50% (*1*). This disease involves gallstones in the bile ducts proximal to the confluence of the right and left hepatic ducts, irrespective of the co-existence of gallstones in the common bile duct (CBD) and/or gallbladder (*2*). In the past, this disease was rare, with a prevalence of 0.6-1.3% (*3*) in the West, but it is increasingly encountered in the West because of greater immigration from Asia (*4-7*).

The etiology of hepatolithiasis has yet to be fully elucidated, although genetic, dietary, and environmental factors are thought to contribute to the disease. Hence, curative management of hepatolithiasis is difficult since treatment depends greatly on fully understanding the mechanism of stone formation. The goal of hepatolithiasis treatment is to resolve ongoing infections, prevent recurrent cholangitis and subsequent hepatic fibrosis, decrease the need for recurrent instrumentation, and prevent progression to cholangiocarcinoma (*2*). Available treatments include medication and surgery. Surgery, including removal of the affected liver segment(s), has been the best treatment thus far. Hepatectomy can remove stones and focal lesions to eliminate the risk of cholangiocarcinoma, strictures, and subsequent bile stasis to provide effective drainage of biliary tract. Complete removal of the diseased lobe or segment is crucial to preventing recurrence and progressive liver disease. The current report describes a system of classifying hepatolithiasis for surgery and firsthand experience managing the disease.

**Address correspondence to:*
Dr. Jiahong Dong, Institute of Hepatobiliary Surgery, Chinese PLA General Hospital, 28 Fuxing Road, Beijing100853, China.
E-mail: xiaobinf@medmail.com.cn

2. Classification of hepatolithiasis for surgery

Classifying surgical candidates or indications for surgery is crucial for a hepatectomy to treat hepatolithiasis to result in the best outcome. Normally, the indications for hepatectomy to treat hepatolithiasis are as follows: *i*) unilobar hepatolithiasis, particularly left-sided; *ii*) atrophy, fibrosis, and multiple abscesses secondary to cholangitis; *iii*) suspicion of concomitant intrahepatic cholangiocarcinoma; and *iv*) multiple intrahepatic stones with biliary strictures that cannot be treated percutaneously or endoscopically (*8*).

The criteria for classification should be based on the pathological characteristics of the biliary tree and hepatic parenchyma. Classification should help to determine treatment strategies and it should be simple and easy to apply. However, there has been no universal classification of hepatolithiasis until now.

Based on previous experience managing hepatolithiasis, the current authors propose a system of classification, designated "Dong's Classification" to determine reasonable surgical approaches to treating hepatolithiasis (Table 1). In this classification, hepatolithiasis is divided into two types, type I and type II. Type I is a localized stone disease with stones located in one (Figure 1A, Type Ia) or both lobes (Figure 1B, Type Ib). Type II is a diffuse stone disease, which is divided into three subtypes: type IIa involves no atrophy of the hepatic parenchyma or stricture of the intrahepatic bile ducts (Figure 2A); type IIb involves segmental atrophy or/and stricture of the intrahepatic bile ducts (Figure 2B); and type IIc involves biliary cirrhosis and portal hypertension (Figure 2C).

The letter "E" represents an additional type of hepatolithiasis with extrahepatic stones. This type is divided into three subtypes: "Ea" representing normal functioning of the sphincter of Oddi; "Eb" representing relaxation of the sphincter of Oddi, and "Ec" representing stricture of the sphincter of Oddi (Figure 3).

3. Hepatectomy to treat hepatolithiasis

Data were collected on patients at the Institute of Hepatobiliary Surgery, Southwest Hospital, and Chinese PLA General Hospital, both of which are leading facilities for biliary surgery in China. Hepatectomy to treat hepatolithiasis was initiated at Southwest Hospital by Prof. Zhiqiang Huang in 1957 and was reported by him in a Chinese medical journal in 1959 (*9*).

Hepatectomy is the best approach to treating hepatolithiasis because it removes stones and also because it removes the strictured bile duct, resects the atrophic portion of the liver, and eliminates the potential presence of cholangiocarcinoma, thus reducing the risk of recurrent stones.

Table 1. Dong's classification of hepatolithiasis for use in determining surgical approaches

Type	Definition or content
Type I	Localized stone disease: unilobar or bilobar.
Type II	Diffuse stone disease.
IIa	No atrophy of the hepatic parenchyma or stricture of the intrahepatic bile ducts.
IIb	Segmental atrophy or/and stricture of the intrahepatic bile ducts.
IIc	Biliary cirrhosis and portal hypertension.
Additional Type E	Extrahepatic stones.
Ea	Normal sphincter of Oddi.
Eb	Relaxation of the sphincter of Oddi.
Ec	Stricture of the sphincter of Oddi.

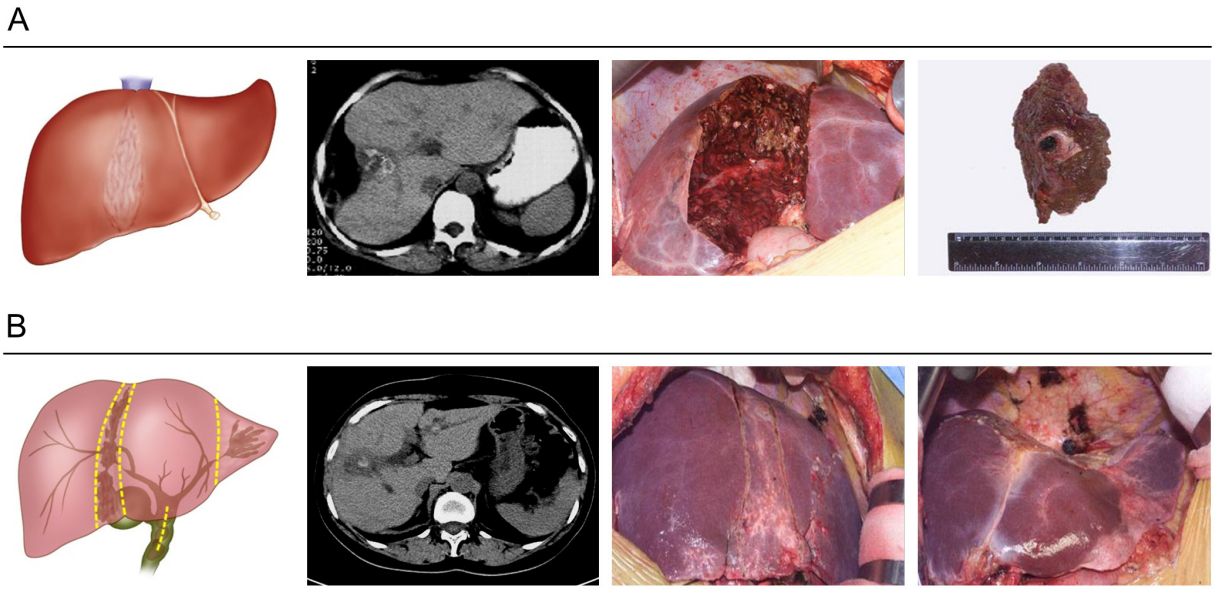


Figure 1. Type I hepatolithiasis. (A), Type Ia. Localized stone disease with stones located in only one lobe. In this case, stones were localized in the atrophic right anterior portion of the liver. Segmentectomy of S5 and S8 was performed; **(B)**, Type Ib. Localized stone disease with stones located in both lobes. In this case, stones were localized in the atrophic right anterior portion and left lateral portion of the liver.

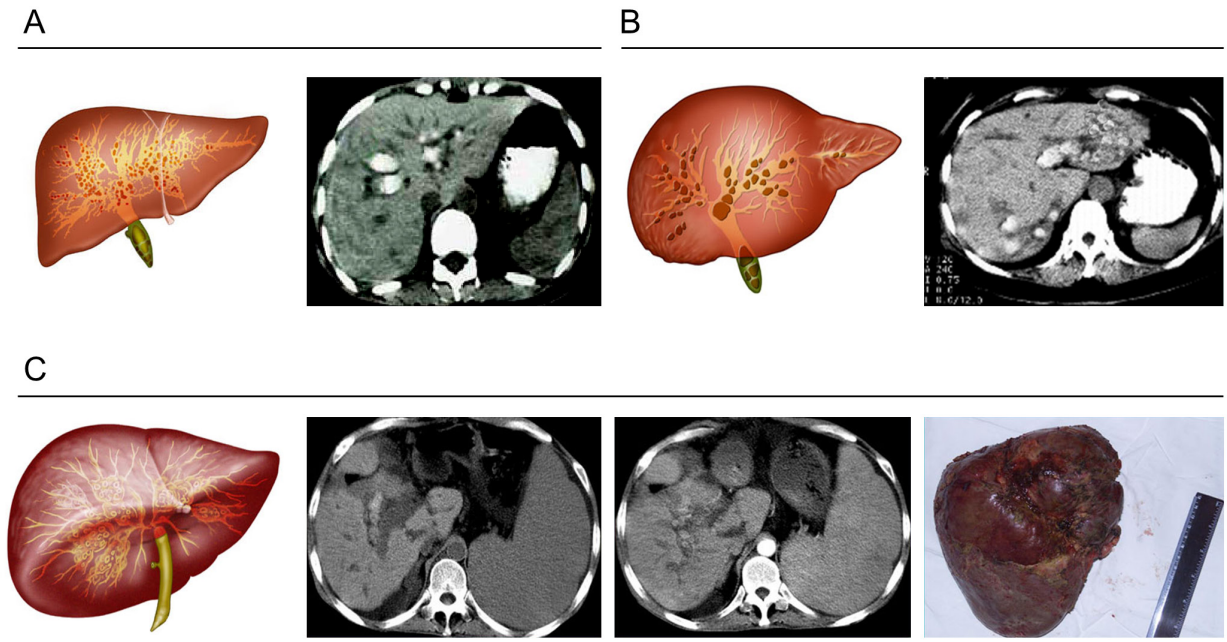


Figure 2. Type II hepatolithiasis. (A), Type IIa. Diffuse stone disease without segmental atrophy or stricture; **(B)**, Type IIb. Diffuse stone disease with segmental atrophy and/or stricture. In this case, stones were located in every liver segment and there was atrophy or biliary stricture of S2, S3, S6, and S7; **(C)**, Type IIc. Diffuse stone disease with secondary biliary cirrhosis. In this case, stones were located in every liver segment and there was biliary cirrhosis and portal hypertension. A liver transplant is needed.

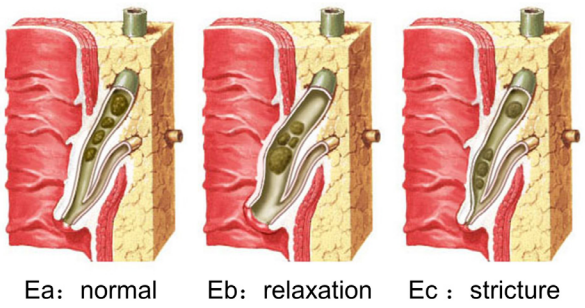


Figure 3. Additional type E hepatolithiasis. Hepatolithiasis with extrahepatic stones. Hepatolithiasis with extrahepatic stones (E) is divided into three subtypes based on functioning of the sphincter of Oddi. Normal function of the sphincter of Oddi is designated "Ea", relaxation of the sphincter of Oddi is designated "Eb", and stricture of the sphincter of Oddi is designated "Ec".

3.1. *Indications for hepatectomy to treat hepatolithiasis*

Based on experience, patients with type I and type IIb hepatolithiasis are the best candidates for hepatectomy. In type I localized stone disease, surgery resects the stone-bearing segments, regardless of where atrophy or a stricture is found (Figure 4). Type II involves a high risk of stone recurrence, so all patients with type II should undergo stone removal along with a Roux-en-Y hepaticojejunostomy (*10,11*) or hepaticocutaneous jejunostomy (*12-15*). Hepatectomy is the best way to resect lesions in patients with type IIb hepatolithiasis and hepatic lesions (*e.g.* segmental atrophy hepatic abscess or cholangiocarcinoma). Patients with type IIc hepatolithiasis consistently have biliary cirrhosis, portal hypertension, and liver failure as well, indicating the need for a liver transplant. Additionally, a hepaticojejunostomy should also be performed to treat "Eb" and "Ec" hepatolithiasis.

3.2. *Outcomes of hepatolithiasis treated by surgery*

From June 1976 to June 2009, 1,930 patients underwent surgery for hepatolithiasis at the Institute of Hepatobiliary Surgery. Of these, 1,175 patients underwent hepatectomy and 755 patients primarily underwent liver-preserving surgery and stone removal.

Perioperative outcomes showed that patients who underwent a hepatectomy had much greater intra-operative bleeding than patients who had stones removed. Hepatectomy was associated with a longer operating time than stone removal. There were no significant differences between the patients in terms of mortality and morbidity. However, the postoperative rate of residual stones was much lower after hepatectomy than after stone removal (Table 2).

The operative morbidity rate was 13.3% for 1,175 patients who underwent a hepatectomy. The most common complication was wound infection, followed by biliary leakage, pleural effusion, pneumonia, stress ulcer, intraabdominal bleeding, and hepatic failure (Table 3).

3.3. *Typical cases where a hepatectomy was used to treat hepatolithiasis*

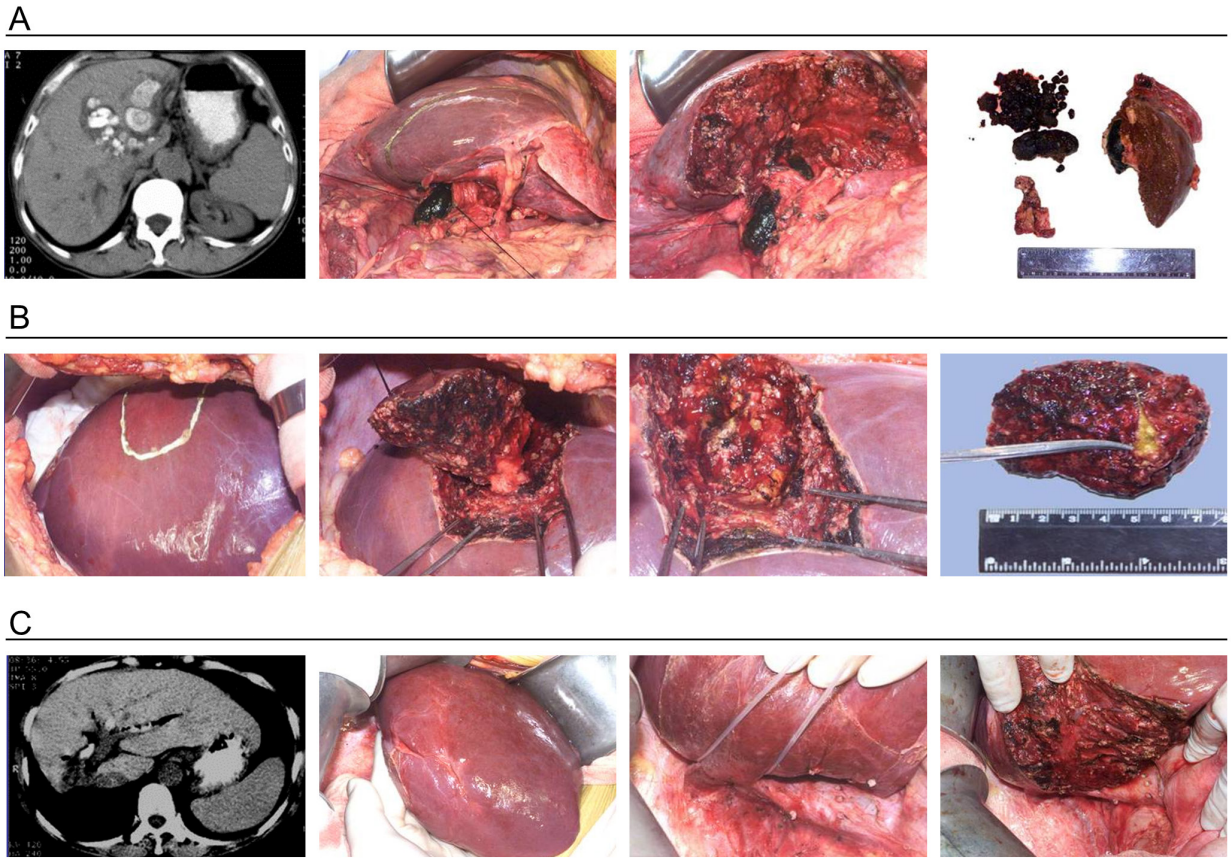


Figure 4. Typical selection of the procedure to treat type I hepatolithiasis. (A), In this case, anatomic left lobectomy was performed to treat type I hepatolithiasis; **(B)**, In this case, segmentectomy of S8 was performed; **(C)**, In this case, a right posterior sectionectomy was performed.

Table 2. Perioperative outcomes for 1,930 patients with hepatolithiasis (June, 1975 – June, 2008)

	HT group (n = 1,175)	SR group (n = 755)	P Value
Operating time (min)*	332 ± 123	289 ± 106	< 0.001
Blood loss (mL)*	717 ± 712	443 ± 510	< 0.001
Postoperative mortality#	3 (2.6%)	1 (1.3%)	1.000
Residual stones#	224 (19.1%)	332 (44.0%)	< 0.001
Perioperative complications#	156 (13.3%)	93 (12.3%)	0.157

HT, Hepatectomy; SR, Stone Removal; *, data are expressed as average ± S.D.; #, data represent cases (ratio).

Table 3. Postoperative complications in 1,175 patients who underwent a hepatectomy (June, 1975 – June, 2008)

Types of Complications	Patients (%)
Wound infection	81 (6.9%)
Biliary leakage	26 (2.2%)
Pleural effusion	21 (1.8%)
Pneumonia	14 (1.2%)
Stress ulcer	13 (1.1%)
Abdominal bleeding	6 (0.5%)
Hepatic failure	3 (0.3%)

3.3.1. *Anatomic left lobectomy, segmentectomy, and sectionectomy for type I hepatolithiasis*

In a case of stones localized in the atrophic left lobe and extrahepatic bile duct, the hepatolithiasis was classified as type I plus Ea, and anatomic left lobectomy was performed and extrahepatic stones were removed (Figure 4A). In a case of a stone localized in atrophic segment VIII, hepatolithiasis was classified as type I, and segmentectomy of S8 was performed (Figure 4B). In a case of stones localized in the atrophic right posterior portion of the liver, anatomic right posterior sectionectomy was performed (Figure 4C).

3.3.2. *Bilateral lobectomy excluding the caudate lobe for type II hepatolithiasis*

From March 2003 to July 2011, 252 patients with type II hepatolithiasis at this Institute underwent surgery. Of these, 12 (4.8%) underwent bilateral lobectomy excluding the caudate lobe. In a typical case, a 39-year-old female had diffusely distributed stones with atrophy in all segments except for the caudate lobe (Figure 5). All 12 of the patients successfully underwent bilateral lobectomy excluding the caudate lobe.

4. Conclusion

Economic development and a more Western lifestyle are associated with a decline in the incidence of

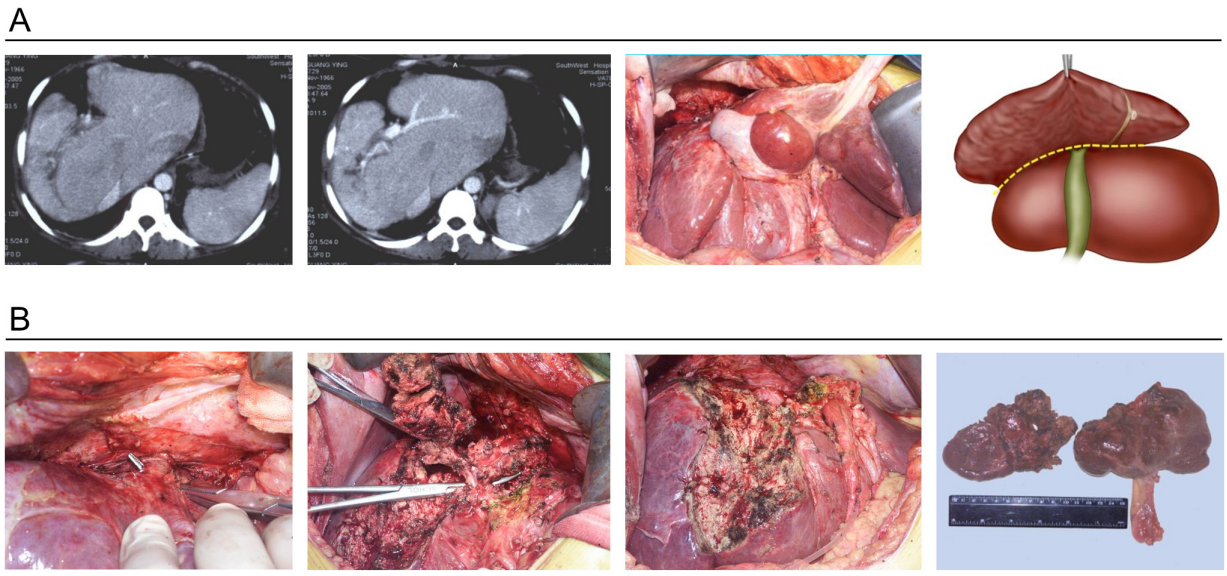


Figure 5. A specific form of bilateral lobectomy excluding the caudate lobe to treat hepatolithiasis. (A), CT images and overall findings; **(B)**, Separation and dissection of liver segments S2-S8, with only segment S1 remaining in the patient.

Table 4. Surgical approaches to treating hepatolithiasis in the literature (*2*)

Series	Years	Primary treatment	No. patients
Uchiyama *et al*. 2007 (*16*)	1971-2006	Hepatectomy	38
Otani *et al*. 1999 (*17*)	1980-1996	Hepatectomy	26
Cheung & Kwok 2005 (*18*)	1989-2003	Hepatectomy	52
Li *et al*. 2006 (*10*)	1992-2002	Hepatectomy	161
Nuzzo *et al*. 2008 (*8*)	1992-2005	Hepatectomy	34
Cheung *et al*. 2003 (*19*)	1993-2001	Percutaneous transhepatic cholangioscopic lithotomy or lithotripsy	79
Li *et al*. 2005 (*20*)	1994-2003	Hepatectomy	46
Kim *et al*. 2006 (*21*)	1994-2004	Hepatectomy	128
Lee *et al*. 2007 (*22*)	2000-2005	Hepatectomy	123
Uenishi *et al*. 2009 (*23*)	1980-2007	Hepatectomy	87
Dong JH *et al*.	1975-2008	Hepatectomy	1,175

hepatolithiasis, but the same is not true for East Asia. Moreover, immigration from the Asian-Pacific region means that this rare but emerging disease will pose a therapeutic challenge to doctors in the West as well.

The optimal management of complex hepatolithiasis remains a very difficult and challenging task for hepatobiliary surgeons. Surgery should be indicated for all cases of hepatolithiasis. The current report describes the largest number of patients with hepatolithiasis treated with a hepatectomy (Table 4) and a novel classification to help in determining surgical strategies. Segmentectomy is an effective treatment for type I and type IIb hepatolithiasis. The caudate lobe is a unique segment that is anatomically separate from the rest of the liver; if the liver were a car, the caudate lobe would be its "spare tire". Finally, better selection of which patients should undergo a hepatectomy will lead to better mid-to long-term outcomes.

Acknowledgements

This project was supported by the Natural Science Foundation of CQ CSTC, No. 2008BB5134 and Project No. 30801113 of NSFC.

References

1. Catena M, Aldrighetti L, Finazzi R, Arzu G, Arru M, Pulitanò C, Ferla G. Treatment of non-endemic hepatolithiasis in a Western country. The role of hepatic resection. Ann R Coll Surg Engl. 2006; 88:383-389.
2. Sakpal SV, Babel N, Chamberlain RS. Surgical management of hepatolithiasis. HPB (Oxford). 2009; 11:194-202.
3. Kayhan B, Akdoğan M, Parlak E, Ozarslan E, Sahin B. Hepatolithiasis: A Turkey experience. Turk J Gastroenterol. 2007; 18:28-32.
4. Park HS, Lee JM, Kim SH, Jeong JY, Kim YJ, Lee KH, Choi SH, Han JK, Choi BI. CT Differentiation of cholangiocarcinoma from periductal fibrosis in patients with hepatolithiasis. AJR Am J Roentgenol. 2006; 187:445-453.
5. Mori T, Sugiyama M, Atomi Y. Gallstone disease: Management of intrahepatic stones. Best Pract Res Clin Gastroenterol. 2006; 20:1117-1137.
6. Pockros PJ. Natural progression of untreated

hepatolithiasis. J Clin Gastroenterol. 2001; 33:95-96.
7. Al-Sukhni W, Gallinger S, Pratzer A, Wei A, Ho CS, Kortan P, Taylor BR, Grant DR, McGilvray I, Cattral MS, Langer B, Greig PD. Recurrent pyogenic cholangitis with hepatolithiasis – The role of surgical therapy in North America. J Gastrointest Surg. 2008; 12:496-503.
8. Nuzzo G, Clemente G, Giovannini I, De Rose AM, Vellone M, Sarno G, Marchi D, Giuliante F. Liver resection for primary intrahepatic stones: A single-center experience. Arch Surg. 2008; 143:570-573; discussion 574.
9. Huang CC. Partial resection of the liver in treatment of intrahepatic stones. Chin Med J. 1959; 79:40-45.
10. Li SQ, Liang LJ, Peng BG, Lai JM, Lu MD, Li DM. Hepaticojejunostomy for hepatolithiasis: A critical appraisal. World J Gastroenterol. 2006; 12:4170-4174.
11. Kusano T, Isa TT, Muto Y, Otsubo M, Yasaka T, Furukawa M. Long-term results of hepaticojejunostomy for hepatolithiasis. Am Surg. 2001; 67:442-446.
12. Leung JW, Yu AS. Hepatolithiasis and biliary parasites. Baillieres Clin Gastroenterol. 1997; 11:681-706.
13. Saing H, Chan KL, Mya GH, Cheng W, Fan ST, Chan FL. Cutaneous stoma in the roux limb of hepaticojejunostomy (hepaticocutaneous jejunostomy): Useful access for intrahepatic stone extraction. J Pediatr Surg. 1996; 31:247-250.
14. Fan ST, Mok F, Zheng SS, Lai EC, Lo CM, Wong J. Appraisal of hepaticocutaneous jejunostomy in the management of hepatolithiasis. Am J Surg. 1993; 165:332-335.
15. Fan ST, Choi TK, Lo CM, Mok FP, Lai EC, Wong J. Treatment of hepatolithiasis: Improvement of result by a systematic approach. Surgery. 1991; 109:474-480.
16. Uchiyama K, Kawai M, Ueno M, Ozawa S, Tani M, Yamaue H. Reducing residual and recurrent stones by hepatectomy for hepatolithiasis. J Gastrointest Surg. 2007; 11:626-630.
17. Otani K, Shimizu S, Chijiiwa K, Ogawa T, Morisaki T, Sugitani A, Yamaguchi K, Tanaka M. Comparison of treatments for hepatolithiasis: Hepatic resection versus cholangioscopic lithotomy. J Am Coll Surg. 1999; 189:177-182.
18. Cheung MT, Kwok PC. Liver resection for intrahepatic stones. Arch Surg. 2005; 140:993-997.
19. Cheung MT, Wai SH, Kwok PC. Percutaneous transhepatic choledochoscopic removal of intrahepatic stones. Br J Surg. 2003; 90:1409-1415.
20. Li X, Shi L, Wang Y, Tian FZ. Middle and long-term clinical outcomes of patients with regional hepatolithiasis after subcutaneous tunnel and hepatocholangioplasty with utilization of the gallbladder. Hepatobiliary Pancreat Dis Int. 2005; 4:597-599.
21. Kim BW, Wang HJ, Kim WH, Kim MW. Favorable outcomes of hilar duct oriented hepatic resection for high grade Tsunoda type hepatolithiasis. World J Gastroenterol. 2006; 12:431-436.
22. Lee TY, Chen YL, Chang HC, Chan CP, Kuo SJ. Outcomes of hepatectomy for hepatolithiasis. World J Surg. 2007; 31:479-482.
23. Uenishi T, Hamba H, Takemura S, Oba K, Ogawa M, Yamamoto T, Tanaka S, Kubo S. Outcomes of hepatic resection for hepatolithiasis. Am J Surg. 2009; 198:199-202.

(Received September 30, 2012; Revised November 3, 2012; Accepted November 9, 2012)

Brief Report

DOI: 10.5582/irdr.2012.v1.4.157

The use of cffDNA in fetal sex determination during the first trimester of pregnancy of female DMD carriers

Dong Wu, Qiaofang Hou, Tao Li, Yan Chu, Qiannan Guo, Bing Kang, Shixiu Liao*

Institute of Medical Genetics, Henan Provincial People's Hospital, Zhengzhou, He'nan, China.

Summary **Chorionic villus sampling (CVS) or amniocentesis for fetal sex determination is generally the first step in the prenatal diagnosis of X-linked genetic disorders such as Duchenne muscular dystrophy (DMD). However, non-invasive prenatal diagnostic (NIPD) techniques such as measurement of cell-free fetal DNA (cffDNA) in maternal plasma are preferable given the procedure-related miscarriage rate of CVS. We determined fetal sex during the first trimester using a quantitative real-time polymerase chain reaction (PCR) assay of cffDNA in pregnant carriers of DMD. The fetal sex was confirmed by amniocentesis karyotype analysis and multiplex ligation-dependent probe amplification (MLPA) at 16 weeks. This procedure may avoid unnecessary CVS or amniocentesis of female fetuses.**

Keywords: Cell-free fetal DNA (cffDNA), non-invasive prenatal diagnostic (NIPD), Duchenne muscular dystrophy (DMD), fetal sex determination

1. Introduction

The first step in the prenatal diagnosis of X-linked genetic disorders like Duchenne muscular dystrophy (DMD) or hemophilia is the determination of fetal sex. Chorionic villus sampling (CVS) and amniocentesis have long been used to determine sex. A female fetus may have a wild-type genotype or be a carrier of DMD, but further genetic analysis is crucial for a male fetus because a male fetus has a 50% change of having DMD. Pregnant carriers risk miscarriage when undergoing an invasive prenatal diagnosis (IPD). Non-invasive prenatal diagnosis (NIPD) is preferable for fetal sex determination during the first trimester since it avoids the unnecessary risks of IPD in pregnant female DMD carriers.

Cell-free fetal DNA (cffDNA) was found in maternal plasma in 1997 (*1*) and its measurement represents a potential form of NIPD. The cffDNA in maternal plasma can be detected as early as 7 weeks. cffDNA comprises about 3-6% of the total cell-free DNA in maternal plasma (*2*). Detecting the *sex-determining region on the Y chromosome* (*SRY*) or other Y chromosome-specific sequences based on cffDNA from maternal plasma is one technique for non-invasive fetal sex determination during the early trimester of pregnancy (*3-5*). Fetal sex can be diagnosed before CVS can be performed. In the current study, cffDNA was measured for fetal sex determination during prenatal diagnosis of DMD. The fetal sex was determined at about 9 weeks of gestation by means of quantitative real-time polymerase chain reaction (PCR) with a taqman probe to detect *SRY* with cffDNA in maternal plasma. If the fetus was male, CVS was performed at 12 weeks followed by DMD and multiplex ligation-dependent probe amplification (MLPA**)** analysis. If the fetus was female, CVS was avoided. Fetal sex was later confirmed by ultrasound at 16 weeks. Whether a female fetus is a carrier or not can be determined after delivery.

2. Materials and Methods

2.1. *Materials*

All study protocols were approved by the Ethics Committee of He'nan Provincial People's Hospital. All of the pregnant women and their partners gave written informed consent and received genetic counseling. MLPA analysis was performed on the fetuses of 15 pregnant women who were DMD carriers.

**Address correspondence to:*
Dr. Shixiu Liao, Institute of Medical Genetics, He'nan Provincial People's Hospital, No.7 Weiwu Road, Jinshui District, Zhengzhou 450003, He'nan, China.
E-mail: ychslshx@yahoo.com.cn

2.2. *Sampling and extraction of cffDNA in maternal plasma*

EDTA blood samples (8 mL) were taken at about 9 weeks of gestation. The blood samples were centrifuged twice at 3,000 g and then at 12,000 g to obtain cell-free plasma. Cell-free DNA was extracted from 2 mL of maternal plasma using the QIAamp blood mini kit (Qiagen, Hilden, Germany). DNA was eluted into 40 μL of solution buffer.

2.3. *Sex determination using quantitative real-time PCR*

Quantitative real-time PCR analysis was performed using an Applied Biosystems 7500 Fast Real-Time PCR System. TaqMan amplification reactions were set up in a reaction volume of 20 μL by use of components (except TaqMan probes and amplification primers) supplied in the TaqMan® Fast Universal PCR Master Mix (2×) (Applied biosystems). TaqMan probes and PCR primers were synthesized by Sangon Biotech (Shanghai, China) Co., Ltd. (Primer1: 5'-TGGCGATT AAGTCAAATTCGC-3'; Primer2: 5'-CCCCCTAGTA CCCTGACAATGTATT-3'; Probe: 5'-(FAM)AGCAGT AGAGCAGTCAGGGAGGCAGA(TAMRA)-3'). Each reaction included 12.5 μL of TaqMan® Fast Universal PCR Master Mix (2×), 300 nM of each amplification primer, and 200 nM of the TaqMan probe. Eight μL of the extracted plasma DNA was used for amplification. Each sample was analyzed twice. The 500 genome equivalent (GE, 6.6 pg of DNA per genome equivalent), 100 GE, and 10 GE were used as positive controls to confirm the sensitivity of the PCR assay. Thermal cycling was initiated with a first denaturation step of 20 s at 95°C and then 40 cycles of 95°C for 3 s and 60°C for 30 s.

2.4. *MLPA analysis of male fetuses*

For male fetuses, DNA was isolated from amniotic fluid cells using the TIANamp Genomic DNA kit (TIANGEN, Beijing, China), and the quality and quantity of DNA was checked using a Nano-Drop 2000 Spectrophotometer. Salsa MLPA kits (P034A2 and P035A2, MRC Holland, Amsterdam, Netherlands) were used to determine whether the fetus had DMD or not. In accordance with the manufacturer's instructions, amplification products were electrophoresed on an ABI 3130 Genetic Analyzer (*6*). Products were then analyzed using Coffalyser v9.2 software.

3. Results and Discussion

3.1. *Fetal sex determination using cffDNA in maternal plasma*

The 15 fetuses studied included 6 males and 9 females, and sex was later confirmed by CVS or ultrasound. Fetal sex was determined by means of quantitative real-time PCR after 9 weeks (Figure 1).

3.2. *MLPA analysis of male fetuses*

As shown in Figure 2, MLPA revealed deletion of exons 3-20 (A, B) and duplication of exons 13-43 (C, D) in male fetuses. Details on the mutations identified are shown in the figure.

The main advantage of measuring cffDNA as part of prenatal diagnostic is that sampling can avoid unnecessary risks associated with conventional techniques of prenatal diagnosis (including CVS and amniocentesis). The mean concentration of cffDNA in maternal plasma was more than 20 times higher than that in the cellular fraction of maternal blood at the same gestational stage. The cellular fraction may be present in maternal plasma for years. cffDNA cannot be detected by enzymolysis a few hours after delivery (*1*), so false-positive results from women who had previously carried a fetus with DMD can be avoided.

cffDNA can be detected in the first trimester of pregnancy. Its measurement is necessary for fetal sex determination since a positive result can allow pregnant women to avoid suffering by terminating a pregnancy early. Non-invasive fetal sex determination using cffDNA can avoid unnecessary CVS and amniocentesis of female fetuses for the prenatal diagnosis of X-linked genetic disorders (*4,7-9*). DMD is one of the most common genetic muscular dystrophies. The onset of symptoms in affected individuals is generally before the age of 5 and most die in the course of the second or third decade of life due to respiratory or heart failure (*10*). Thus, prenatal diagnosis is crucial for families of

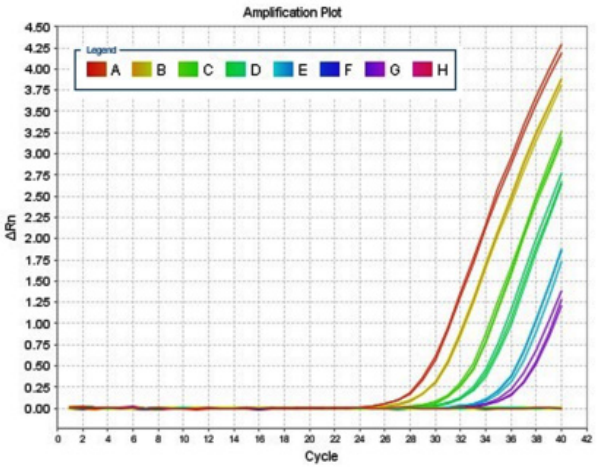


Figure 1. Quantitative real-time PCR. Amplification plots obtained using quantitative real-time PCR for the *SRY* gene. A: 500GE; B: 100GE; C, D, and E: positive samples; F: negative controls; G: 10GE; H: negative samples. The X-axis denotes the cycle number of a quantitative PCR reaction. The Y-axis denotes ΔRn, which is the fluorescence intensity over the background.

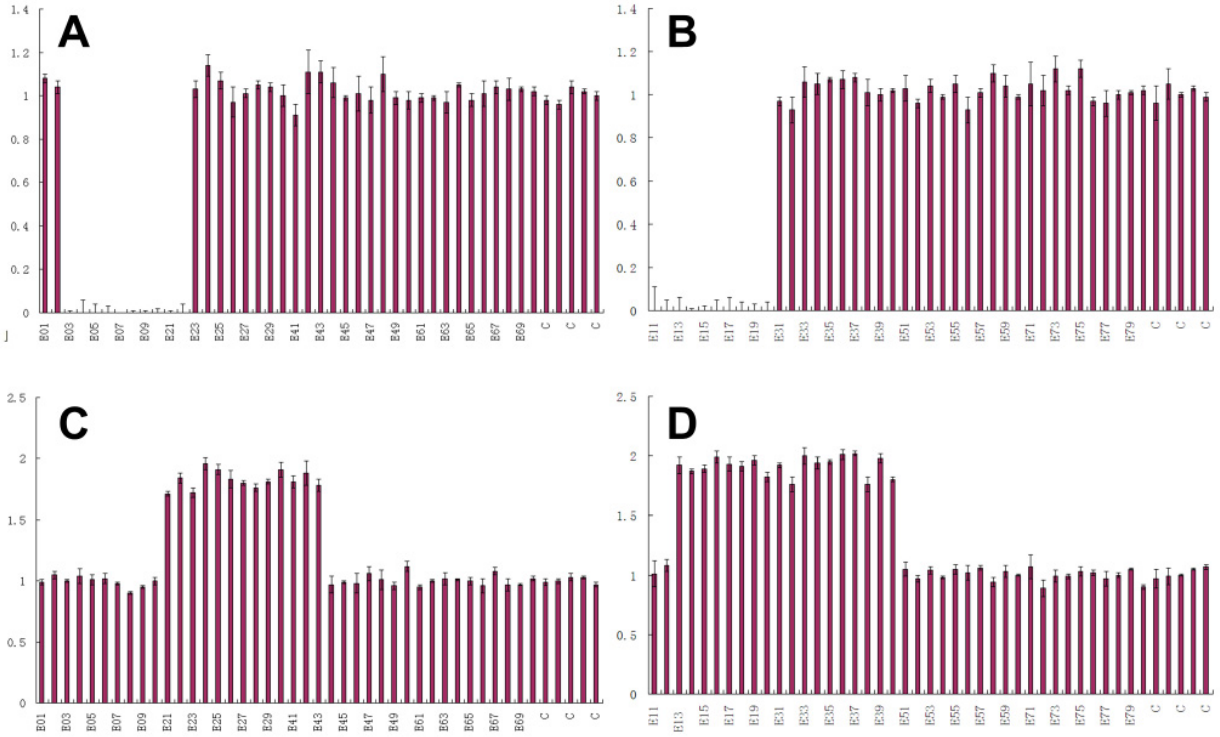


Figure 2. MLPA analysis of DMD. (A), The result for 034 indicates deletion of exons 3-10, 21, and 22; **(B)**, The result for 035 indicates deletion of exons 11-20; **(C)**, The result for 034 indicates duplication of exons 21-30 and 41-43; **(D)**, The result for 035 indicates duplication of exons 13-20 and 31-40.

carriers.

Fetal DNA analysis using maternal plasma would be most useful in the detection of paternally-inherited fetal mutations or autosomal recessive genetic disorders where the father and mother carry different mutations (*11-14*). Increased amounts of fetal DNA may also be found in instances of conditions associated with placental damage, such as pre-eclampsia (*15,16*). Recent studies showed that cffDNA could be used in prenatal screening for fetal chromosomal disorders (trisomy 18, trisomy 13, and Down syndrome) (*17,18*).

The main difficulty of using cffDNA in NIPD lies in the low concentration of cffDNA and presence of a larger quantity of background maternal DNA in plasma, so sensitive and specific techniques like the use of a real-time TaqMan system are needed. In conclusion, cffDNA could be used for fetal sex determination in the first trimester of pregnancy to screen for gender-specific inherited disorders while avoiding an unnecessary CVS.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 81170581).

References

1. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, Wainscoat JS. Presence of fetal DNA in maternal plasma and serum. Lancet. 1997; 350:485-487.
2. Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, Wainscoat JS, Johnson PJ, Chang AM, Hjelm NM. Quantitative analysis of fetal DNA in maternal plasma and serum: Implications for noninvasive prenatal diagnosis. Am J Hum Genet. 1998; 62:768-775.
3. Lewis C, Hill M, Skirton H, Chitty LS. Fetal sex determination using cell-free fetal DNA: Service users' experiences of and preferences for service delivery. Prenat Diagn. 2012; 32:735-741.
4. Miura K, Higashijima A, Shimada T, Miura S, Yamasaki K, Abe S, Jo O, Kinoshita A, Yoshida A, Yoshimura S, Niikawa N, Yoshiura K, Masuzaki H. Clinical application of fetal sex determination using cell-free fetal DNA in pregnant carriers of X-linked genetic disorders. J Hum Genet. 2011; 56:296-299.
5. Kim SY, Lim JH, Park SY, Kim MY, Choi JS, Ryu HM. Non-invasive prenatal determination of fetal gender using QF-PCR analysis of cell-free fetal DNA in maternal plasma. Clin Chim Acta. 2012; 413:600-604.
6. Verma PK, Dalal A, Mittal B, Phadke SR. Utility of MLPA in mutation analysis and carrier detection for Duchenne muscular dystrophy. Indian J Hum Genet. 2012; 18:91-94.
7. Kolialexi A, Tounta G, Apostolou P, Vrettou C, Papantoniou N, Kanavakis E, Antsaklis A, Mavrou A. Early non-invasive detection of fetal Y chromosome sequences in maternal plasma using multiplex PCR. Eur J Obstet Gynecol Reprod Biol. 2012; 161:34-37.
8. Jin S, Lin XM, Law H, Kwek KY, Yeo GS, Ding C. Further improvement in quantifying male fetal DNA in maternal plasma. Clin Chem. 2012; 58:465-468.
9. Hill M, Compton C, Lewis C, Skirton H, Chitty LS.

Determination of foetal sex in pregnancies at risk of haemophilia: A qualitative study exploring the clinical practices and attitudes of health professionals in the United Kingdom. Haemophilia. 2012; 18:575-583.
10. Emery AE. The muscular dystrophies. Lancet. 2002; 359:687-695.
11. Long XJ, Long GF, Lin WX. Noninvasive prenatal diagnosis of Hb Bart's hydrops fetus using cell-free fetal DNA in maternal plasma. Zhonghua Xue Ye Xue Za Zhi. 2009; 30:175-178.
12. Tounta G, Vrettou C, Kolialexi A, Papantoniou N, Destouni A, Tsangaris GT, Antsaklis A, Kanavakis E, Mavrou A. A multiplex PCR for non-invasive fetal RHD genotyping using cell-free fetal DNA. In Vivo. 2011; 25:411-417.
13. Paterlini Bréchot P, Mouawia H, Saker A. Non-invasive prenatal diagnosis of cystic fibrosis. Arch Pediatr. 2011; 18:111-118.
14. Yan TZ, Mo QH, Cai R, Chen X, Zhang CM, Liu YH, Chen YJ, Zhou WJ, Xiong F, Xu XM. Reliable detection of paternal SNPs within deletion breakpoints for non-invasive prenatal exclusion of homozygous α-thalassemia in maternal plasma. PLoS One. 2011; 6:e24779.
15. Jakobsen TR, Clausen FB, Rode L, Dziegiel MH, Tabor A. High levels of fetal DNA are associated with increased risk of spontaneous preterm delivery. Prenat Diagn. 2012; 32:840-845.
16. Hahn S, Rusterholz C, Hösli I, Lapaire O. Cell-free nucleic acids as potential markers for preeclampsia. Placenta. 2011; 32 (Suppl):S17-20.
17. Palomaki GE, Deciu C, Kloza EM, Lambert-Messerlian GM, Haddow JE, Neveux LM, Ehrich M, van den Boom D, Bombard AT, Grody WW, Nelson SF, Canick JA. DNA sequencing of maternal plasma reliably identifies trisomy 18 and trisomy 13 as well as Down syndrome: An international collaborative study. Genet Med. 2012; 14:296-305.
18. Ashoor G, Syngelaki A, Wagner M, Birdir C, Nicolaides KH. Chromosome-selective sequencing of maternal plasma cell-free DNA for first-trimester detection of trisomy 21 and trisomy 18. Am J Obstet Gynecol. 2012; 206:322.e1-5.

(Rceived August 23, 2012; Accepted October 13,2012)

Original Article

DOI: 10.5582/irdr.2012.v1.4.161

Study and analysis of the state of rare disease research in Shandong Province, China

Heng Zhao[1,2], Yazhou Cui[1], Xiaoyan Zhou[1], Jingxiang Pang[1], Xiumei Zhang[2], Shuangqing Xu[1], Jinxiang Han[1,*]

[1] *Shandong Medicinal Biotechnology Center, Key Laboratory for Biotech Drugs of the Ministry of Health, Key Laboratory for Rare Disease of Shandong Province, Shandong Academy of Medical Sciences, Ji'nan, Shandong, China;*
[2] *Ji'nan University Shandong Academy of Medical Sciences College of Life Science and Medicine, Ji'nan, Shandong, China.*

Summary **As the world's most populous country, China has the world's largest number of rare disease groups in terms of prevalence. However, the country has no system of registering cases of most rare diseases, so there is very little documented information on the epidemiology of those diseases. The purpose of this study was to study the state of rare disease research and survey doctors in Shandong Province regarding their level of awareness of rare diseases. Types of rare diseases and numbers of cases were tallied and their geographical distribution over the decades was analyzed. Eight hundred and twenty-four doctors in tertiary hospitals and maternity and child care hospitals were surveyed by questionnaire. Data were descriptively analyzed and a map of disease distribution was created. Articles about rare diseases were retrieved from the Chinese Biomedical Literature Database to provide pertinent data. This study yielded 5,749 cases of 323 different types of rare diseases. The survey found that doctors lack awareness of research on rare diseases. An authoritative and information-rich platform for rare disease research is urgently needed. Key steps are to study epidemiological and statistical techniques and then obtain available data to provide a basis for the definition and regulation of rare diseases in China.**

Keywords: Rare diseases, awareness survey, descriptive analysis

1. Introduction

Rare diseases are also known as "orphan diseases", but there is no satisfactory definition of rare diseases around the world. In the United States of America (USA), a rare disease is defined as a disease that affects fewer than 200,000 individuals, but in Japan the number is 50,000 and in Australia it is 2,000. The European Union (EU) definition is less than 5 in 10,000. The World Health Organization (WHO) defines a rare disease as all pathological conditions affecting 0.65-1 out of every 1,000 inhabitants (*1*). These numbers clearly relate to the population sizes of these countries, but even adjusting for that, the definitions vary from about 1 to 8 in 10,000 (*2*). Data on rare diseases are constantly collected and updated through the combined efforts of government, patient organizations, and medical and scientific institutions. Many organizations, such as orphanet (*http://www.orpha.net*) in the EU, have showed that these data play an important role in areas such as the prevention and treatment of rare diseases, policy-making, medical research, and social welfare.

Study and regulation of rare diseases has progressed worldwide, and this is especially true in the USA and EU. The USA adopted important legislation on rare disease and orphan drugs in 1983 that has successfully promoted investment in research and development (R&D) of new pharmaceutical products to treat rare diseases. Similar legislation was also enacted in Australia in 1997 and in the EU in 1999. This legislation explicitly recognized the unmet need for targeted treatments for rare diseases and it created

**Address correspondence to:*
Dr. Jinxiang Han, Shandong Academy of Medical Sciences, No. 18877 Jing-shi Road, Ji'nan, 250062, Shandong, China.
E-mail: samshjx@sina.com

regulatory pathways and incentives for manufacturers to develop orphan drugs (*3-5*). In Asia, Japan, South Korea, and Taiwan have established systematic economic and regulatory incentives to encourage R&D of drugs for rare diseases.

China is also actively promoting regulation of rare diseases, but these diseases have not been covered by the national health system and special legislation on orphan drugs was only recently enacted (*6*). Given its large population, China probably has a large number of patients with rare diseases according to the definition of the WHO. However, there are still no official data on "the prevalence of rare diseases, their variety, and the number of cases of rare diseases". A crucial step is to collect data on rare diseases in China.

Shandong Province is one of China's most populous provinces, the sixth census recorded its population as 95,793,065, which represents 7.2% of China's total population. The province started researching rare diseases early on and it created a rare disease prevention and control association. At present, the goal of that association is to establish a platform for rare disease diagnosis and information. This project is also supported by the provincial government and has a good research foundation. Shandong has a varying terrain, rich mineral resources, traditional, historical, and cultural backgrounds, both an industrial and an agricultural economy, various occupations and socioeconomic levels, and relatively developed medical technology. It epitomizes China. Data on rare diseases from Shandong Province can be used as a national analogue to a certain extent and will help to study rare diseases and formulate responses nationally. Thus, the current study examined the state of rare disease research and level of awareness of rare diseases among doctors in Shandong. This study also tallied the types of rare diseases and number of cases and analyzed their geographical distribution over the decades.

2. Methods

2.1. *Data collection*

A questionnaire was sent to 824 doctors in a total of 103 tertiary hospitals and municipal maternity and child care hospitals in 17 cities of Shandong Province. The questionnaire (Table 1) consisted of three parts. The first dealt with background information, including name, specialty, phone number, e-mail, position, title, education, years of experience, and department. The second part was a survey on awareness and recommendations for prevention of rare diseases. The third part dealt with information on which types of rare diseases were present and the number of cases encountered. There were 472 responses from doctors at 59 hospitals (219 doctors at 3 hospitals has never encountered a rare disease and thus did not answer the questionnaire), for a total response rate of 57.28% (472/824). Here, the 253 responses (53.60% of valid responses) from doctors who had encountered a rare disease are analyzed.

Also studied were articles on rare diseases from the Database of Chinese Biomedicine Literature (*http://*

Table 1. Portions of the questionnaire

Topic	Item	Control
Survey on awareness of rare diseases among doctors and recommendations for their prevention	1. How did you learn of rare diseases?	a) Newspapers and the Internet b) Professional lectures c) Related training d) Other
	2. If you encounter a rare disease that you are unfamiliar with, what you will do?	a) Treat in accordance with clinical experience b) Ask a veteran physician c) Look for further information d) Refer to another department or hospital
	3. What is the best way to improve the diagnosis and treatment of rare diseases?	a) Establish a network and create a platform at the provincial level to diagnose, treat, and study rare diseases b) Provide consultations and improve the efficiency of diagnosis and treatment of rare diseases at the provincial level
	4. Which step is the most important in setting up a network to study rare diseases?	a) Assemble cases and conduct scientific research b) Participate in joint consultations and improve diagnosis and treatment c) Establish molecular diagnostic techniques d) Carry out collaborative research and joint studies on rare diseases
	5. Have you ever conducted research on rare diseases?	a) Yes b) No
	6. Have you ever published papers about rare diseases?	a) Yes b) No
	7. Have you ever cared for patients with rare diseases?	a) Yes b) No
Disease informationare	1. Disease 2. Number of cases	

sinomed.imicams.ac.cn) with a principal author residing in Shandong Province from January 1978 to January 2012. The retrieval strategy used was "all fields: disease and author affiliation: Shandong". These articles were then narrowed down to eliminate case reports, resulting in 409 papers.

2.2. *Data processing and analysis*

SPSS 20.0 was used to input and manage data, and then random sampling was used to ensure the accuracy of data. If there were abnormal data or missing values, they were corrected in accordance with the original data.

Descriptive statistical analysis in the form of frequency distribution analysis was used and data were summarized to describe the data characteristics. Charts were then drawn with SPSS and Excel.

Maps with shades of color reflecting the number of cases of rare diseases and number of articles published in each city were created with ArcMap 10.0 and SPSS 20.0.

3. Results

This study found 5,749 cases of 323 different types of rare diseases in Shandong Province. The survey of doctors yielded 293 types and 4,068 cases and the Chinese Biomedical Literature Database yielded 50 types and 1,681 cases. Figure 1 shows data on rare diseases according to descriptive analysis. The ten most prevalent rare diseases were fibrous dysplasia of bone, congenital hypothyroidism, Marfan's syndrome, infectious mononucleosis, hemophilia, osteogenesis imperfecta, multiple osteochondroma, synpolydactyly, phenylketonuria, and neurofibromatosis.

Figure 2 displays the geographic distribution of rare disease cases and articles in shades of color. Binzhou, Ji'nan, Qingdao, and Linyi had more cases than other locations (Figure 2A). Cities with the higher number of articles on rare diseases published in the Chinese Biomedical Literature Database were Ji'nan, Qingdao, and Weifang (Figure 2B). Dezhou, Laiwu, and Rizhao had few cases of rare disease and few articles on those diseases in the database.

The data in Figure 3 shows the awareness of rare diseases among doctors and the recommendations to prevent those diseases. As shown in the figure, doctors learned about rare diseases through professional seminars, clinical consultation, magazines, newspapers, the Internet, and related training, with professional seminars being the most frequent method of learning (Figure 3A). When doctors encountered a patient with a rare disease, 46% choose to seek more information, 27% referred the patient, 20% consulted a veteran physician and the remaining 7% treated the patient based on their clinical experience (Figure 3B). Seventy percent of respondents felt that the best way to improve the diagnosis and treatment of rare diseases was to establish a network and platform to diagnose, treat, and study rare diseases (Figure 3C). According to respondents, important steps in establishing such a network are assembling cases and conducting scientific research, participating in joint consultations and improving diagnosis and treatment, establishing molecular diagnostic techniques, and carrying out

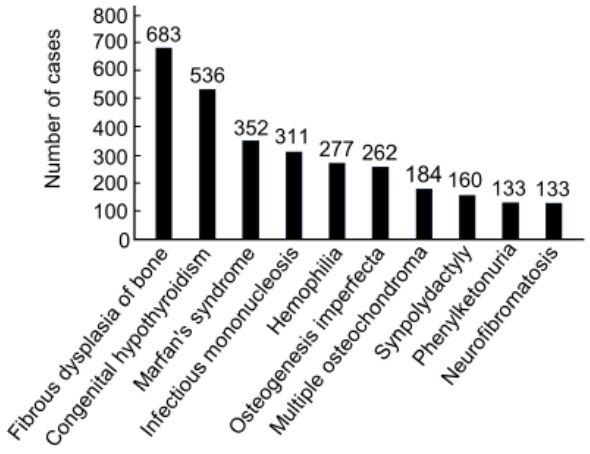


Figure 1. Ten most prevalent rare diseases in terms of the number of cases. Columns represent the number of cases.

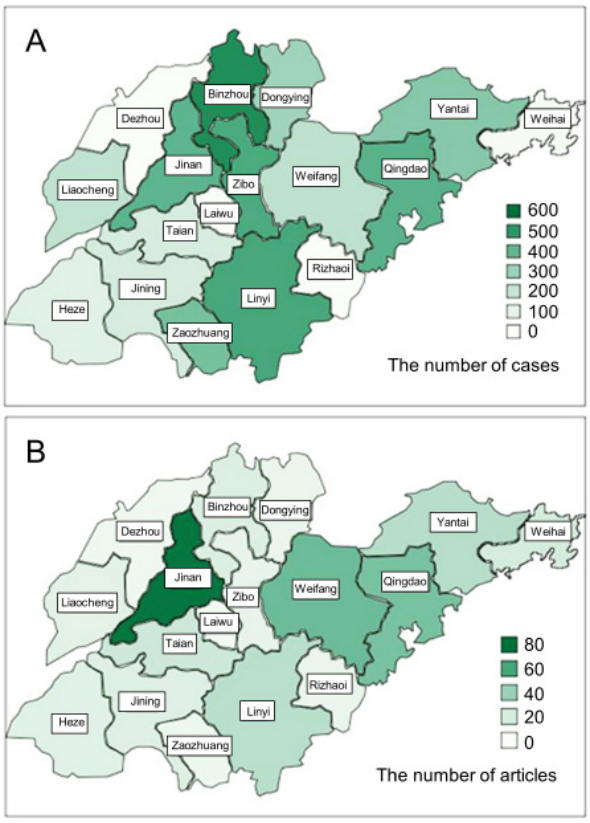


Figure 2. Geographical distribution of rare diseases on those diseases. (A), Shown is the distribution of cases in each city; **(B)**, Shown are locations where articles on rare diseases are published. Color shades indicate degree.

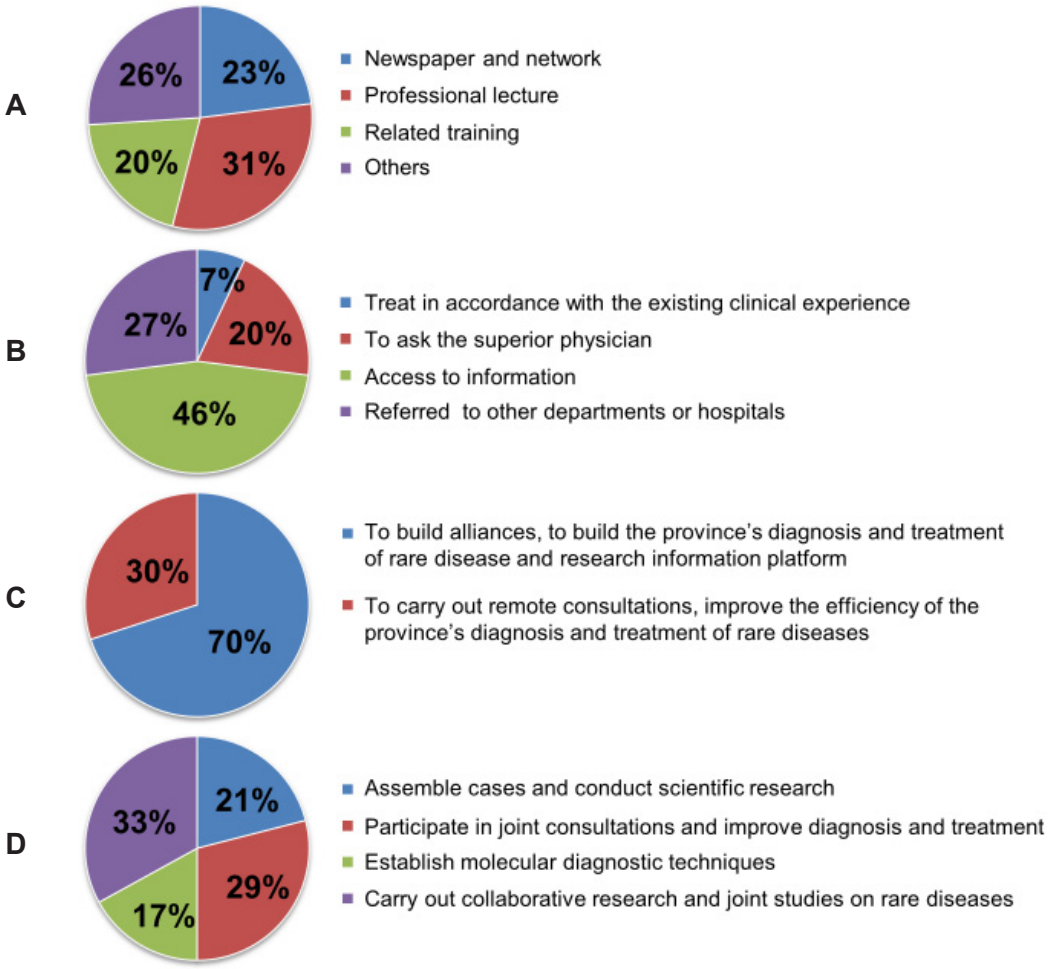


Figure 3. Data on awareness of rare diseases among doctors and recommendations. The pie chart shows the proportion of answers. **(A)**, Responses to the question "How did you learn of rare diseases?"; **(B)**, Responses to the question "If you encounter a rare disease that you are unfamiliar with, what you will do?"; **(C)**, Responses to the question "What is the best way to improve the diagnosis and treatment of rare diseases?"; **(D)**, Responses to the question "Which step is the most important in setting up a network to study rare diseases?".

collaborative research and joint studies on rare diseases (Figure 3D).

Figure 4 shows the low degree of emphasis on rare disease research among doctors. Forty-four of 247 doctors had carried out research on rare diseases, 33 of 250 had published an article on a rare disease, and 10 of 249 had participated in a project on a rare disease.

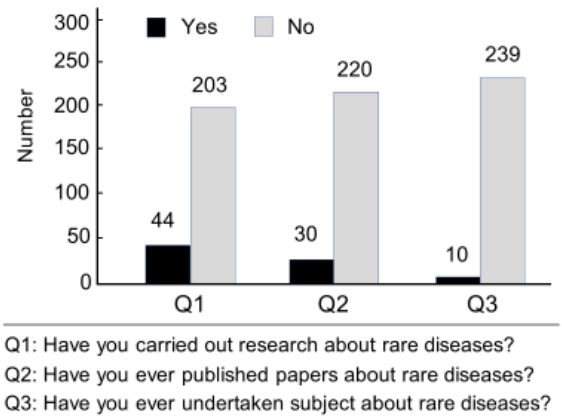


Figure 4. Data on rare disease research. Columns indicate the number of responses.

4. Discussion

As the world's most populous country, China has the world's largest number of rare disease groups in terms of prevalence. However, the country has no system of registering cases of most rare diseases, so there is very little documented information on the epidemiology of those diseases (*7,8*). The current study tallied the ten most prevalent rare diseases in Shandong Province, but this list did not match the diseases described in other articles. Bibliographic data published in 2011 by Orphanet (*http://www.orpha.net*) in Europe showed that the ten most prevalent rare diseases were Klippel-Trenaunay-Weber syndrome, Whipple disease, incontinentia pigmenti, Aicardi syndrome, CADASIL, Li-Fraumeni syndrome, Silver-Russell syndrome,

Castleman disease, cutis marmorata telangiectatica congenital, and Mobius syndrome. The prevalence of rare diseases may differ in different countries or different areas, so a database specific to China must be created for future research.

A survey of rare disease awareness among doctors found that they learn about rare diseases in many ways, though none is authoritative and standards and effective programs for diagnosis and treatment of rare diseases are lacking. A similar survey was conducted in Europe, but patients were surveyed. The survey was part of a long-term study that began in March 2004 by sending a questionnaire to 18,000 patients to ask about experience being diagnosed with a rare disease in 17 countries. The survey results highlighted the dilemma of rare diseases: lack of information, lack of appropriate medical training, difficulties with access to care, and subsequent loss of patient confidence in the health care system and the medical profession. The survey authors put forward solutions including reference centers, databases for the exchange of information, DNA and tissues banks, and networks of professionals (*9*). The establishment of a rare disease network and information exchange platform is a pressing matter. At the same time, work must also be done to assemble cases and conduct scientific research, participate in joint consultations and improve diagnosis and treatment, establish molecular diagnostic techniques, and carry out collaborative research and joint studies on rare diseases.

The current study noted a very serious problem: the low degree of emphasis on rare disease research among doctors. Rare disease research received little attention and also involved a low degree of cooperation. The current survey had a response rate below 60%, and fewer than 55% of respondents had ever encountered a rare disease. The problem may be due to doctors' specialties, interests, or level of knowledge, but definitive conclusions cannot be reached due to the lack of data. Further study and analysis will be done to determine the factors influencing attention to rare diseases among doctors and increase that attention.

The study of rare diseases in China is still in its infancy. China is also actively promoting regulation of intractable and rare diseases. Some rare disease websites and online databases to register cases have been set up. A number of centers have been established to offering counseling on rare diseases in major Chinese cities like Beijing and Shanghai. Moreover, in Shanghai patients with 12 rare diseases recently became eligible for partial reimbursement, and some special orphan drugs for children are now covered by insurance, but these diseases have not been covered by the national health system and special legislation on orphan drugs was only recently enacted. China still lags far behind the US, EU, Japan, and other countries and regions in terms of orphan drug legislation (*6,10,11*). Key steps are to examine epidemiological and statistical techniques and then obtain available data to provide a basis for the definition and regulation of rare diseases in China.

The current study has several limitations. There was, for example, substantial bias in the data collection process and the survey was conducted using only descriptive analysis. Rare disease cases and articles have different geographic distributions, but definitive reasons for these differences were not apparent, so data need to be collected and in-depth analysis needs to be performed to determine whether there are significant differences in rare diseases seen in different cities and hospital departments.

5. Conclusion

Shandong Province had at least 5,749 cases of 323 different types of rare diseases, and the distribution of these diseases differed in different cities. Few doctors in the province had ample knowledge about rare diseases and there is insufficient emphasis on the treatment and research of rare diseases. An authoritative and information-rich platform for rare disease research is urgently needed, and the low degree of emphasis on rare disease research among doctors must soon be improved. Key steps are to examine epidemiological and statistical techniques and then obtain available data to provide a basis for the definition and regulation of rare diseases in China.

Acknowledgements

This work was supported by the Health Department of Shandong Province. The authors wish to thank Mrs. Wang Hui and Mrs. Sun Xiaoyun (the chief of the Science and technology education and international cooperation department of Shandong Province) and doctors from all of the tertiary hospitals and municipal maternity and child care hospitals in Shandong Province who participated in this study.

References

1. Lavandeira A. Orphan drugs: Legal aspects, current situation. Haemophilia. 2002; 8:194-198.
2. Aronson JK. Rare diseases and orphan drugs. Br J Clin Pharmaco. 2006; 61:243-245.
3. Villa S, Compagni A, Reich MR. Orphan drug legislation: Lessons for neglected tropical diseases. Int J Health Plann Manage. 2009; 24:27-42.
4. Taruscio D, Capozzoli F, Frank C. Rare diseases and orphan drugs. Ann Ist Super Sanita. 2011; 47:83-93.
5. Rinaldi A. Adopting an orphan. EMBO Rep. 2005; 6:507-510.
6. Song PP, Gao JJ, Inagaki Y, Kokudo N, Tang W. Intractable and rare diseases research in Asia. Biosci Trends. 2012; 6:48-51.
7. Wang JB, Guo JJ, Yang L, Zhang YD, Sun ZQ, Zhang YJ. Rare diseases and legislation in China. Lancet. 2010;

375:708-709.
8. Zhang YJ, Wang YO, Li L, Guo JJ, Wang JB. China's first rare-disease registry is under development. Lancet. 2011; 378:769-770.
9. EURORDIS. Survey of the delay in diagnosis for 8 rare diseases in Europe (Eurordiscare 2). *http://archive.eurordis.org/article.php3?id_article=454* (access February 1, 2012)
10. Song PP, Gao JJ, Inagaki Y, Kokudo N, Tang W. Rare diseases, orphan drugs, and their regulation in Asia: Current status and future perspectives. Intractable Rare Dis Res. 2012; 1:3-9.
11. Han JX, Cui YZ, Zhou XY. Rare diseases research in China: Opportunities, challenges, and solutions. Intractable Rare Dis Res. 2012; 1:10-12.

(Reveived August 21, 2012; Revised October 8, 2012; Accepted October 12, 2012)

Case Report

DOI: 10.5582/irdr.2012.v1.4.167

Henoch-Schönlein purpura associated with a neuroblastoma: Report of one case and a review of the literature

Qiaoli Dong[1, 2], Shanshan Cao[1, 3], Hongwen Zhang[1,*], Hui Geng[1]

[1] *Department of Pediatrics, Peking University First Hospital, Beijing, China;*
[2] *Department of Pediatrics, Affiliated Hospital of Hebei University, Baoding, China;*
[3] *Health Center, Beijing Entry-exit Inspection and Quarantine Bureau, Beijing, China.*

Summary **Malignancies such as solid tumors and hematologic malignancies can often induce or be associated with Henoch-Schönlein purpura (HSP) in older males but not in children. Described here is the case of a 5-year-old boy who clinically presented with HSP. An imaging study of the abdomen revealed a right retroperitoneal neoplasm that histopathology postoperatively confirmed to be a neuroblastoma. Malignancies are sometimes associated with HSP mostly in older males, though children are affected, albeit rarely. Thus, all patients with HSP must be carefully examined to identify or exclude an underlying disease.**

Keywords: Henoch-Schönlein purpura (HSP), neuroblastoma, malignancy

1. Introduction

Henoch-Schönlein purpura (HSP) is the most common vasculitic disease affecting children. HSP is a multisystem immunoglobulin A-mediated vasculitis with a self-limited course that affects the skin, joints, gastrointestinal tract, and kidneys (*1,2*). HSP has many causes, including infections, drugs, foods, and malignant tumors. Many malignancies, such as solid tumors and hematologic malignancies, are reported to induce or be associated with HSP. Such tumors include carcinoma of the lung, bronchus, esophagus, stomach, intestine, breast, kidney, prostate, and thyroid (*3-12*) while hematologic malignancies include non-Hodgkin lymphoma, Hodgkin disease, multiple myeloma, myeloproliferative disease, and myelodysplastic syndrome (*10,11,13-17*).

However, patients with HSP associated with a malignancy are mostly older males with a mean age of 60 years (*7,8,10,11*). Neuroblastomas are the most common extracranial solid tumors in children, but there are no reports of children with HSP associated with a neuroblastoma as has been reported here.

**Address correspondence to:*
Dr. Hongwen Zhang, Department of Pediatrics, Peking University First Hospital, No.1, Xi An Men Da Jie, Beijing 100034, P. R. China.
E-mail: zhanghongwen@yeah.net

2. Case report

A 5-year-old boy with a weight of 19.5 kg (75 percentile), height of 110.0 cm (50 percentile), and blood pressure of 100/75 mmHg presented with numerous purpuras, bilateral knee joint pain, and abdominal pain without bloody diarrhea at almost the same time. The boy had no upper respiratory tract infections or other precipitating factors prior to the onset of those symptoms. A physical examination on admission found numerous flat or palpable purpuras that were typical of HSP in both lower extremities. There were no signs or abnormal findings upon physical examination of the abdomen. No lymphadenopathy and hepatosplenomegaly was evident. The boy was diagnosed with HSP.

Laboratory results such as urine output, renal function, prothrombin time, complements C3 and C4, anti-double stranded DNA antibodies, antinuclear antibodies, and anti-smooth muscle antibodies, C-reaction protein, and antistreptolysin O were all normal. The boy was treated with vitamin C, calcium gluconate, cimetidine, and dipyramidole. Joint pain and abdominal pain improved but purpuras did not change. A routine abdomen ultrasound two weeks after the diagnosis of HSP revealed a right adrenal-occupying lesion (4 cm × 6 cm) and magnetic resonance imaging suggested a neoplasm. Further laboratory tests revealed higher levels of vanillylmandelic acid (VMA) and

homovanillic acid (HVA) in the urine. Histopathology confirmed the neoplasm to be an adrenal neuroblastoma (stage II, American Children's Oncology Group staging system, CCSG). HSP completely resolved soon after surgery, and continued chemotherapy was given using the OPEC schedule (vincristine [O], cisplatin [P], etoposide [E], and cyclophosphamide [C]). HSP did not recur during a follow-up of 12 months.

3. Discussion

The patient experienced purpura and joint and abdominal pain without kidney involvement, fulfilling the diagnostic criteria for HSP (*18*). Abdominal ultrasound and magnetic resonance imaging revealed a right retroperitoneal neoplasm that histopathology postoperatively confirmed to be a neuroblastoma. HSP was associated with a neuroblastoma in this 5-year-old boy. Moreover, neuroblastoma was an incidental finding diagnosed by the routine abdomen ultrasound; the patient had no abdominal signs or abnormal physical findings on admission.

Malignancies are known to cause vasculitis like HSP. In 2006, Zurada *et al*. (*11*) reviewed literature on adult malignancy-associated HSP from around the world, and they found a total of 31 cases. Patients were overwhelmingly male (94%) with a mean age of 60 years and presented predominantly with solid tumors (61%) and secondly with hematologic malignancies (39%). The most frequent tumors were lung cancer (n = 8), multiple myeloma (n = 5), prostate cancer (n = 5), and non-Hodgkin lymphoma (n = 3). The majority of patients (55%) developed HSP within 1 month of cancer diagnosis or detection of metastases. Their findings were similar to those in a report by Pertuiset *et al.* (*8*). In 2009, Mitsui *et al.* (*10*) reported 23 cases of HSP in patients with underlying malignant tumors. HSP was thought to be closely associated with a tumor in nine patients, and seven of the nine exhibited new metastatic lesions or died due to underlying cancer within 1-32 months. Based on these reports, HSP associated with a malignancy is characterized by: *i*) patients who are mainly older males (over 40-60 years: over 85%); *ii*) development within 1-3 months of diagnosis or metastasis of a neoplasm; *iii*) causes are mostly solid tumors (over 60%), and especially carcinoma of the lung, followed by hematologic malignancies (about 40%); and *iv*) development in the absence of a precipitating factor. This suggests that adults, and especially older men who present with unexplained HSP, should be evaluated for an occult neoplasm (*5,6*), while patients with a known history of malignancy who present with HSP should be evaluated for metastatic disease (*11*). A skin biopsy is an important way to determine the underlying pathology in adult HSP (*17*). Malignancies induce or are associated with HSP mostly in older males, but children can also be affected, albeit rarely. Funato *et al*. (*19*) reported acute lymphoblastic leukemia mimicking HSP in a 3-year-old boy.

Neuroblastomas are the most common extracranial solid tumors in children, accounting for about 8%-10% of all pediatric tumors (*20,21*). That said, there are no reports of HSP associated with a neuroblastoma or other malignancy in children. The current case is the world's first case of HSP associated with a neuroblastoma.

Tumors are known to be one of the causes of vasculitis (*8,10,22*). Vasculitis is reported to occur during the course of malignancies in 2.3%-8% of patients (*23*). The incidence of vasculitis in cancer is estimated to be 1 in 1,800 for hemopathies and 1 in 80,800 for solid tumors (*24*). The relationship between vasculitis and malignancy remains unclear: *e.g.* fortuitous association, paraneoplastic syndrome, or neoplasms induced by immunosuppressive drugs prescribed to treat vasculitis, and so on (*25*). HSP is an allergic vasculitis disease caused by an immunologic mechanism (*26-29*). Neoplasm antigens such as paraneoplastic antibodies or abnormally produced IgA lead to the formation of immune complexes that induce the lesions of HSP (*10*).

The development of HSP and a neuroblastoma at the same time in the current patient is curious. Maybe both diseases developed independently or maybe HSP was a paraneoplastic syndrome of the neuroblastoma. However, neuroblastomas are known to induce other forms of vasculitis such as Kawasaki disease (*30*). In the current patient, HSP was likely to be induced by the neuroblastoma. The first reason for this conjecture is because the neuroblastoma appeared to develop prior to HSP given to the size of the neuroblastoma and the course of HSP. Second, there were no precipitating factors before the onset of HSP and no relapse during 12 months of follow-up. However, genetic studies of the neuroblastoma, *e.g.* studies of the *N-Myc* gene and paraneoplastic antibodies, were not performed, and neither was a skin biopsy.

In conclusion, many malignancies may cause HSP, but in older males HSP is mostly caused by solid tumors. Reported here is the first case of HSP associated with a neuroblastoma in a 5-year-old boy. Epidemiological studies are needed to determine the association between HSP and malignancy in children.

References

1. Gedalia A. Henoch-Schonlein purpura. Curr Rheumatol Rep. 2004; 6:195-202.
2. Reamy BV, Williams PM, Lindsay TJ. Henoch-Schönlein purpura. Am Fam Physician. 2009; 80:697-704.
3. Hughes RA, Bottomley DM, Keat AC, Drury A. Henoch-Schonlein purpura occurring in association with carcinoma of the breast. Eur J Med. 1993; 2:310-312.
4. Maestri A, Malacarne P, Santini A. Henoch-Schönlein syndrome associated with breast cancer. A case report. Angiology. 1995; 46:625-627.

5. Weiler-Bisig D, Ettlin G, Brink T, Arnold W, Glatz-Krieger K, Fischer A. Henoch-schönlein purpura associated with esophagus carcinoma and adenocarcinoma of the lung. Clin Nephrol. 2005; 63:302-304.
6. Frigui M, Kechaou M, Ben Hmida M, Kamoun K, Khanfir A, Frikha M, Hachicha J, Bahloul Z. Adult Schönlein-Henoch purpura associated with epidermoid carcinoma of the lung. Nephrol Ther. 2009; 5:201-204.
7. Flynn AN, du Prey B, Al Ardati H, Raman M, Lemaire J. Adult-onset malignancy-associated Henoch-Schönlein purpura. Scand J Rheumatol. 2011; 40:325-326.
8 Pertuiset E, Liote F, Launay-Russ E, Kemiche F, Cerf-Payrastre I, Chesneau AM. Adult Henoch-Schönlein purpura associated with malignancy. Semin Arthritis Rheum. 2000; 29:360-367.
9 Negri M, Peruzy AD. On a case of Schöenlein-Henoch syndrome in a subject with thyroid adenocarcinoma. Rass Fisiopatol Clin Ter. 1961; 33:331-337.
10. Mitsui H, Shibagaki N, Kawamura T, Matsue H, Shimada S. A clinical study of Henoch-Schönlein Purpura associated with malignancy. J Eur Acad Dermatol Venereol. 2009; 23:394-401.
11. Zurada JM, Ward KM, Grossman ME. Henoch-Schönlein purpura associated with malignancy in adults. J Am Acad Dermatol. 2006; 55(Suppl 5):S65-70.
12. Blanco R, González-Gay MA, Ibáñez D, Alba C, Pérez de Llano LA. Henoch-Schönlein purpura as a clinical presentation of small cell lung cancer. Clin Exp Rheumatol. 1997; 15:545-547.
13. Blanco P, Denisi R, Rispal P, Deminière C, Pellegrin JL, Leng B, Aparicio M. Henoch-Schönlein purpura associated with segmental and focal proliferative glomerulonephritis in a patient with Hodgkin's disease. Nephrol Dial Transplant. 1999; 14:179-180.
14. Conte G, Conte FJ, Ojeda JM, Araos D, Poniachik J, Murray G, Flores C. Severe Henoch-Schönlein purpura in a patient with multiple myeloma. Rev Med Chil. 2000; 128:1255-1260.
15. Saurina A, Botey A, Solé M, Vera M, Pou M, Torras A, Darnell A. Henoch-Schönlein purpura nephritis associated with coagulase-negative staphylococci sepsis in a patient with myeloma. Nephrol Dial Transplant. 2001; 16:2441-2442.
16. Fox MC, Carter S, Khouri IF, Giralt SA, Prieto VG, Nash JW, Hymes SR. Adult Henoch-schönlein purpura in a patient with myelodysplastic syndrome and a history of follicular lymphoma. Cutis. 2008; 81:131-137.
17. Tabata R, Tabata C, Namiuchi S, Terada M, Yasumizu R, Okamoto T, Nagai T. Adult T-cell lymphoma mimicking Henoch-Schönlein purpura. Mod Rheumatol. 2007; 17:57-62.
18. Mills JA, Michel BA, Bloch DA, *et al*. The American College of Rheumatology 1990 criteria for the classification of Henoch-Schönlein purpura. Arthritis Rheum. 1990; 33:1114-1121.
19. Funato M, Kaneko H, Kubota K, Ozeki M, Kanda K, Orii K, Kato Z, Fukao T, Kondo N. Pediatric acute lymphoblastic leukemia mimicking Henoch-Schönlein purpura. Pediatr Int. 2011; 53:766-768.
20. Malmstrom-Groth A. Cerebellar encephalopathy and neuroblastoma. Eur Neurol. 1972; 7:95-100.
21. Aydin GB, Kutluk MT, Buyukpamukcu M, Akyuz C, Yalcin B, Varan A. Neurological complications of neuroblastic tumors: Experience of a single center. Childs Nerv Syst. 2010; 26:359-365.
22. Watts RA, Lane S, Scott DG. What is known about the epidemiology of the vasculitides? Best Pract Res Clin Rheumatol. 2005; 19:191-207.
23. Blanco R, Martinez-Taboada VM, Rodriguez-Valverde V, García-Fuentes M. Cutaneous vasculitis in children and adults. Associated diseases and etiologic factors in 303 patients. Medicine (Baltimore). 1998; 77:403-418.
24. Greer JM, Longley S, Edwards NL, Elfenbein GJ, Panush RS. Vasculitis associated with malignancy. Experience with 13 patients and literature review. Medicine (Baltimore). 1988; 67:220-230.
25. Fain O, Hamidou M, Cacoub P, *et al*. Vasculitides associated with malignancies: Analysis of sixty patients. Arthritis Rheum. 2007; 57:1473-1480.
26. Eleftheriou D, Dillon MJ, Brogan PA. Advances in childhood vasculitis. Curr Opin Rheumatol. 2009; 21:411-418.
27. Tizard EJ, Hamilton-Ayres MJ. Henoch Schönlein purpura. Arch Dis Child Educ Pract Ed. 2008; 93:1-8.
28. Roberts PF, Waller TA, Brinker TM, Riffe IZ, Sayre JW, Bratton RL. Henoch-Schönlein purpura: A review article. South Med J. 2007; 100:821-824.
29. Tullus K, Marks SD. Vasculitis in children and adolescents: Clinical presentation, etiopathogenesis, and treatment. Paediatr Drugs. 2009; 11:375-380.
30. Ohta S, Narita T, Kato H,Taga T, Takeuchi Y. A patient with Kawasaki disease who developed acute urinary retention due to pelvic neuroblastoma. Eur J Pediatr. 2002; 161:631.

(Received August 26, 2012; Revised October 21, 2012; Accepted October 27, 2012)

Author Index (2012)

Subject Index (2012)

Editorial

Policy Forums

Reviews

Brief Reports

Original Articles

Case Report

Commentaries

Letter

IRDR
Intractable & Rare Diseases Research

Guide for Authors

1. Scope of Articles

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

2. Submission Types

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.

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 100 references. Mini reviews are also accepted.

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 2,000 words in length (excluding 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).

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.

3. Editorial Policies

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.

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 signed 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 http://www.irdrjournal.com/downcentre.php (Download Centre).

Copyright: A signed JOURNAL PUBLISHING AGREEMENT (JPA) form must be provided by post, fax, or as a scanned file before acceptance of the article. Only forms with a hand-written signature are accepted. This copyright will ensure the widest possible dissemination of information. A form facilitating transfer of copyright can be downloaded by clicking the appropriate link and can be returned to the e-mail address or fax number noted on the form (Please visit Download Centre). 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.

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 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: A one-paragraph abstract consisting of no more than 250 words must be included. The abstract should briefly state the purpose of the study, methods, main findings, and conclusions. Abbreviations must be kept to a minimum and non-standard abbreviations explained in brackets at first mention. References should be avoided in the abstract. 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. 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.

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 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. All color figures will be reproduced in full color in the online edition of the journal at no cost to authors.

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 http://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 authord for the publication of their article except for reprints.

(As of February 2012)

Editorial and Head Office:

Pearl City Koishikawa 603
2-4-5 Kasuga, Bunkyo-ku
Tokyo 112-0003, Japan
Tel: +81-3-5840-9968
Fax: +81-3-5840-9969
E-mail: office@irdrjournal.com

IRDR
Intractable & Rare Diseases Research

JOURNAL PUBLISHING AGREEMENT (JPA)

Manuscript No.:

Title:

Corresponding Author:

The International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) is pleased to accept the above article for publication in Intractable & Rare Diseases Research. The International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) reserves all rights to the published article. Your written acceptance of this JOURNAL PUBLISHING AGREEMENT is required before the article can be published. Please read this form carefully and sign it if you agree to its terms. The signed JOURNAL PUBLISHING AGREEMENT should be sent to the Intractable & Rare Diseases Research office (Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku, Tokyo 112-0003, Japan; E-mail: office@irdrjournal.com; Tel: +81-3-5840-9968; Fax: +81-3-5840-9969).

1. Authorship Criteria

As the corresponding author, I certify on behalf of all of the authors that:

1) The article is an original work and does not involve fraud, fabrication, or plagiarism.
2) The article has not been published previously and is not currently under consideration for publication elsewhere. If accepted by Intractable & Rare Diseases Research, the article will not be submitted for publication to any other journal.
3) The article contains no libelous or other unlawful statements and does not contain any materials that infringes upon individual privacy or proprietary rights or any statutory copyright.
4) I have obtained written permission from copyright owners for any excerpts from copyrighted works that are included and have credited the sources in my article.
5) All authors have made significant contributions to the study including the conception and design of this work, the analysis of the data, and the writing of the manuscript.
6) All authors have reviewed this manuscript and take responsibility for its content and approve its publication.
7) I have informed all of the authors of the terms of this publishing agreement and I am signing on their behalf as their agent.

2. Copyright Transfer Agreement

I hereby assign and transfer to IACMHR Co., Ltd. all exclusive rights of copyright ownership to the above work in the journal Intractable & Rare Diseases Research, including but not limited to the right 1) to publish, republish, derivate, distribute, transmit, sell, and otherwise use the work and other related material worldwide, in whole or in part, in all languages, in electronic, printed, or any other forms of media now known or hereafter developed and the right 2) to authorize or license third parties to do any of the above.

I understand that these exclusive rights will become the property of IACMHR Co., Ltd., from the date the article is accepted for publication in the journal Intractable & Rare Diseases Research. I also understand that IACMHR Co., Ltd. as a copyright owner has sole authority to license and permit reproductions of the article.

I understand that except for copyright, other proprietary rights related to the Work (*e.g.* patent or other rights to any process or procedure) shall be retained by the authors. To reproduce any text, figures, tables, or illustrations from this Work in future works of their own, the authors must obtain written permission from IACMHR Co., Ltd.; such permission cannot be unreasonably withheld by IACMHR Co., Ltd.

3. Conflict of Interest Disclosure

I confirm that all funding sources supporting the work and all institutions or people who contributed to the work but who do not meet the criteria for authors are acknowledged. I also confirm that all commercial affiliations, stock ownership, equity interests, or patent-licensing arrangements that could be considered to pose a financial conflict of interest in connection with the article have been disclosed.

Corresponding Author's Name (Signature):

Date: