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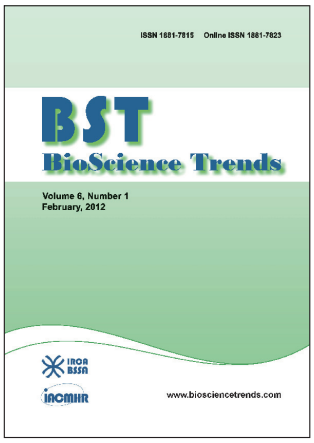

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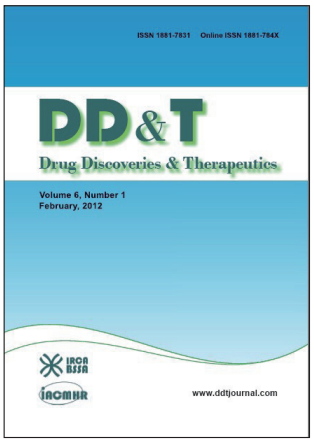

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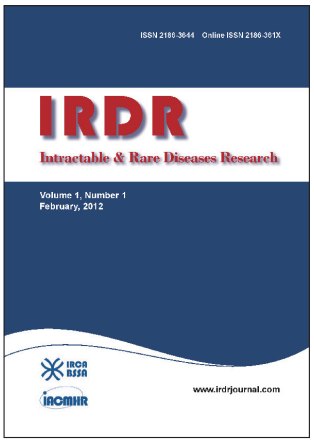

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Editorial and Head Office

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
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Hopes for intractable and rare diseases research

Wei Tang*

Department of Surgery, The University of Tokyo Hospital, Tokyo, Japan.

February 28, 2013 marks the sixth international "Rare Disease Day". On and around this day, hundreds of patient organizations from more than 60 countries and regions worldwide plan to host awareness campaigns in line with this year's theme, "Rare Disorders without Borders". Public awareness of intractable and rare diseases has heightened in recent decades. Much progress has also been made worldwide, such as specific legislation to encourage discovery and development of orphan drugs in the United States (US), the European Union (EU), and some parts of Asia. However, there are still many gaps in knowledge with regard to therapeutic tools and strategies. Intractable and rare diseases cause patients substantial physical suffering, psychological despair, and economic hardships due to bleak therapeutic outcomes and the lack of practical support in everyday life. The features of intractable and rare diseases and the increasing number of types of identified diseases make these diseases an important public health issue and a challenge to medical care worldwide. The following are specific aspects of research on intractable and rare diseases that need to be promptly promoted.

An International Classification of Diseases (ICD) code to promote the definition and classification of intractable and rare diseases

Intractable diseases, or "nanbyo" (literally "hard-to-treat diseases" in Japanese), mainly refer to rare diseases that have resulted mostly from unidentifiable causes and/or a lack of clearly established or curative treatments. According to the World Health Organization (WHO), rare diseases are rare and often debilitating or even life-threatening diseases or conditions with a prevalence of 0.65-1‰. The conventional view is that rare diseases as a whole affect around 10% of individuals worldwide, but the definition and categorization of rare diseases differ slightly by region. In the US, rare diseases are defined as diseases that affect fewer than 200,000 Americans (prevalence of < 0.75‰), while stipulated prevalence rates in other regions are < 0.5‰ in the EU, fewer than 2,000 patients (prevalence of < 0.11‰) in Australia, fewer than 50,000 patients (prevalence of < 0.4‰) in Japan, fewer than 20,000 patients (prevalence of < 0.4‰) in South Korea, or a prevalence of < 0.1‰ in Taiwan. The current outlook for identification of a specific rare disease and estimation of the true burden of rare diseases is bleak given the lack of proper classification and coding of rare diseases. Currently, there is no special coding system for rare diseases. The current ICD code 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, preventing assessment of the economic and social effects of rare diseases. Fortunately, the good news is that the European Rare Disease Task Force of the Health and Consumers Protection Directorate General of the European Commission has set up a working group to collaborate with the WHO on the ICD-10, and the group is considering all other existing classifications to provide the rare disease community with a uniform system. A revised ICD code is urgently needed to both promote the definition and classification of intractable and rare diseases and to obtain accurate epidemiological data on these diseases at the national and international levels.

Specific legislation to encourage discovery and development of orphan drugs

Currently, orphan drugs – the medicinal products intended for the diagnosis, prevention, or treatment of rare diseases – are a major facet of how rare diseases are dealt with. In the past few decades, many countries have realized that orphan drugs will not lead to substantial sales under normal market conditions because of the high costs and risks of drug development, insufficient knowledge of the pathophysiological mechanisms of rare diseases that the drugs diagnose or treat, and difficulties in conducting clinical trials with small patient populations and a small potential market. Therefore, specific legislation to encourage the discovery and development of orphan drugs was enacted in many

**Address correspondence to:*
Dr. Wei Tang, Department of Surgery, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: tang-sur@h.u-tokyo.ac.jp

countries and regions, including the US in 1983, Japan in 1993, Australia in 1997, the EU in 1999, Taiwan in 2000, and South Korea in 2003. Incentives include financial subsidies, market exclusivity, tax credits, fee waivers, fast track approval, and protocol assistance, resulting in substantial improvements in the treatment of patients with a range of rare diseases. While China is actively preparing to regulate and encourage the development of orphan drugs, it still lags far behind the US, the EU, Japan, and other countries and regions with orphan drug legislation. Evidence has shown that all of the incentives have successfully encouraged the development of new pharmaceutical products to treat rare diseases. Prior to 2010, 352 orphan drugs were approved in the US, helping an estimated 12 million Americans, compared to only 10 such drugs in the decade preceding the Orphan Drug Act (1983). Similarly, 720 drugs had received orphan drug designation from the European Medicines Agency (EMA) and 63 designated orphan medicinal products have been authorized for marketing in the EU. Furthermore, data have shown that an average of 15 new orphan drugs are approved annually in the US and 10-12 new orphan drugs are approved annually in the EU. Thus, China and other countries without orphan drug legislation need to promptly establish domestic legislative regulations and incentives to encourage discovery and development of orphan drugs.

Government-funded special biomedical research programs to enhance basic and applied research on intractable and rare diseases

Biomedical research on intractable and rare diseases has provided insights into the pathologies of these diseases and revealed their underlying mechanisms. Such work may ultimately reveal possible avenues to therapeutics. Moreover, once biomedical research identifies suitable drug candidates and becomes more translational, it will garner industry attention, potentially leading to safe and effective orphan drugs. In Western countries, many research centers or projects have been established to support special biomedical research programs on rare diseases and development of orphan drugs, such as the Office of Rare Diseases Research (ORDR) established in the US in 1993 within the National Institutes of Health (NIH) and the Rare Disease Task Force (RDTF) established in EU in 2004 within the European Commission Public Health Directorate. In Asian countries, biomedical research on intractable and rare diseases has made great advances in Japan due to the systematic Specified Disease Treatment Research Program established in 1972 with the support of the Ministry of Health, Labor, and Welfare. As a result, special research programs and research grants from government sources to study 130 diseases increased to 10 billion Japanese yen in 2010. Recently, 214 diseases were designated for a second round of special research programs. In China, support for special biomedical programs on intractable and rare disease research comes mainly from the National Natural Science Foundation of China (NSFC). Data showed that 366 projects (involving 32 rare diseases) were funded by the NSFC from 1999 to 2007 with total funding of 89.358 million RMB and annual funding of about 10 million RMB, accounting for just 1/10th of similar funding in the US. Special biomedical research programs that enhance basic and applied research on intractable and rare diseases would benefit patients through better diagnosis and more treatment choices. Government-funded special biomedical research programs need to be promptly implemented in China and other countries to promote research on intractable and rare diseases.

Patients' advocacy organizations and disease registry networks to provide vast information on intractable and rare diseases

In recent years, progress has been made in the dissemination of knowledge and information by established patients' advocacy organizations, such as the National Organization for Rare Disorders (NORD) in the US and the European Organization for Rare Diseases (EURORDIS) in Europe, but the delay in diagnosis and treatment is still a huge challenge to cope with. A survey of 18,000 individuals found that 25% of patients waited for 5-30 years before being correctly diagnosed and 40% of patients were diagnosed incorrectly before they were correctly diagnosed. Furthermore, clinical studies on orphan drugs also face challenges due to the small size of the trial population and the fact that patients are often geographically dispersed. Disease registry networks need to be established to promote epidemiological and basic research and improve the clinical outcome for patients with intractable and rare diseases. In Western countries, some web-based resources, such as the Rare Diseases Clinical Research Network (RDCRN) in the US and the Orphanet in Europe, have been established in order to facilitate collaboration on clinical outcomes and to share accumulated experience so that patients with intractable and rare diseases are not delayed access to orphan drugs. More patients' advocacy organizations and disease registry networks need to be promptly established to facilitate interaction among patients, clinicians, researchers, the pharmaceutical industry, and governmental bodies with the ultimate goal of promoting intractable and rare disease research worldwide.

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Review

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Research progress on flavonoids isolated from traditional Chinese medicine in treatment of Alzheimer's disease

Jianjun Gao[1,*], Yoshinori Inagaki[2], Yang Liu[3]

[1] *Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;*
[2] *The Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan;*
[3] *Department of Anesthesia, Shandong Provincial Qianfoshan Hospital, Ji'nan, Shandong, China.*

Summary **Alzheimer's disease (AD) is a severe condition in aging countries. The currently used drugs including donepezil, rivastigmine, galantamine, and memantine are effective in managing the symptoms. However, they are hardly capable of preventing, halting, or reversing the disease. In the long history of development of traditional Chinese medicine, much experience has accumulated and is summarized in treatment of diseases that correspond to the concept of AD. In recent years, exploration of natural active ingredients from medicinal herbs for treatment of AD has attracted substantial attention. Some flavonoids have been revealed to have a variety of biological actions such as scavenging free radicals, inhibiting neuron apoptosis, and nurturing neuronal cells that constitute the basis for treatment of AD. In this article, we review recent research progress on flavonoids isolated from traditional Chinese medicine against AD and their underlying mechanisms.**

Keywords: Ginkgo flavonoids, soy isoflavones, puerarin, total flavonoids of Baical Skullcap stem and leaf, liquiritin, apigenin

1. Introduction

Alzheimer's disease (AD) is characterized by progressive deterioration in intellect including memory and cognitive functions. It is the most common type of dementia among older people, accounting for 50-75% of all dementia cases (*1*). The number of AD patients was estimated at 36 million in 2010 and will triple in the world by 2050 (*2*). In China, this figure is estimated at 9 million currently and the prevalence rate of AD in the population over the age of 60 years is 2.43% (*3,4*). Proportionate increases over the next forty years in the number of people with AD will be much steeper in China since it is witnessing the aging of society in which the population over the age of 60 years will account for approximately 31% (about 400 million calculated on the current population base) of the whole population by the year of 2050 (*5*). These epidemiological data have painted a less than optimistic outlook in prevention and treatment of this disease in the world, especially in those countries with a rapidly aging society such as China.

**Address correspondence to:*
Dr. Jianjun Gao, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.
E-mail: gao-tky@umin.ac.jp

The currently approved drugs for treatment of AD, *e.g.* donepezil, rivastigmine, galantamine, and memantine, aim to either inhibit acetylcholine esterase to increase the levels of the neurotransmitter acetylcholine, or antagonize *N*-methyl-D-aspartic acid (NMDA)-type glutamate receptors to prevent aberrant neuronal stimulation (*6,7*). These medicines, however, exhibit modest and transient effects in improving disease manifestation and could hardly prevent, halt, or reverse the disease (*2*). The typical course of AD lasts for a decade or so, from the mildest stage when the symptoms like memory problems appear to the most severe stage when the patients must depend on others for basic activities of daily living and finally die in a completely helpless state. The long duration of AD and shortage of effective or curative treatments bring an enormous emotional and financial burden on patients, their families and society.

In the past several decades, much research has been done to evaluate the anti-AD effects of natural agents isolated from traditional Chinese medicines from perspectives such as scavenging free radicals, inhibiting lipid peroxidation, suppressing neuronal apoptosis, enhancing the function of cholinergic neurons, and improving behavioral abnormalities in experimental

animal models (*8-10*). Flavonoids are a series of compounds that are spread widely in higher plants and ferns and have attracted much attention due to their various biological actions (*11*). The characteristic chemical structures of these compounds is two benzene rings with hydroxyl groups linked by a three-carbon chain (*11*). The most commonly known biological action of flavonoids is their antioxidant activity, which could be understood from the reduction properties of phenol hydroxyls in the chemical structures. That said, compounds of this type exhibit various pharmacological effects and clinical efficacies that may not be solely related to their anti-oxidative activities, such as effects on the vascular system, inflammatory response, and estrogen-like effects (*11*). These actions of flavonoids constitute the underlying basis for their anti-AD effects. In this article, we review recent research progress on flavonoids isolated from traditional Chinese medicine against AD and their underlying mechanisms.

2. Pathological basis of AD

The presence of extracellular amyloid plaques, intracellular neurofibrillary tangles (NFTs), and loss of neurons and synapses in the cerebral cortex and certain subcortical regions in the brain are the main features of AD (*2*). A great deal of evidence indicates that the onset of AD is probably the consequences of complex interactions among genetic, environmental, and lifestyle factors (*12*). The pathogenesis of AD has been revealed to correlate with the following aspects.

2.1. *Genetic factors*

AD has been demonstrated to be related to mutations or polymorphisms of at least four genes, including *amyloid precursor protein* (*APP*), *presenilin* (*PS*)-*1*, *PS-2*, and *apolipoprotein E4* (*APOE4*) located at chromosomes 21, 14, 1, and 19, respectively (*13*). Early-onset (< 60 years) familial AD, which probably accounts for less than 1% of AD cases, was found to be caused by mutations in *APP*, *PS-1*, and *PS-2* genes (*14,15*). It was demonstrated that genetic abnormality occurred in at least one of these three genes in the early-onset familial AD. Late-onset (> 60 years) familial and sporadic AD, which accounts for most AD cases, has been genetically linked to *APOE4* which has a gene-dosage effect on increasing the risk and lowering the age of onset of the disease (*16,17*). In addition, genetic defects of *PS-1* and *APOE4* were usually discovered in sporadic AD (*12*).

2.2. *Aggregation and accumulation of amyloid-β (Aβ) in the brain*

The amyloid plaques of AD brains largely consist of Aβ protein, which is a 39-42 amino acid protein derived from its parent protein, APP, by proteolytic cleavage at the β- and γ-secretase cleavage sites (*12*). The amyloid cascade hypothesis suggested Aβ is the pathogenic factor and drives the progression of this disease. The aggregation and accumulation of Aβ, which may result from increased production of Aβ, decreased degradation by Aβ-degrading enzymes, or reduced clearance across the blood-brain barrier, gave rise to plaques which induced neurodegeneration and finally led to the clinical dementia syndrome typical of AD (*2*). It was found that nonfibrillar assemblies of Aβ such as Aβ dimers, trimers, and larger oligomers are more pathogenic than insoluble Aβ fibrils found in amyloid plaques and monomeric Aβ (*2*). The neurotoxic activities of Aβ were expressed through a mechanism that induces intracellular generation of reactive oxygen species (ROS), lipid peroxidation, calcium overload, and eventually neuronal death (*18-22*).

2.3. *Formation of NFT in neurons*

Besides the abnormal accumulation of amyloid plaques, another pathologic feature of AD is intracellular formation of NFT which are primarily made up of aggregated tau protein bearing abnormal posttranslational modifications, including increased phosphorylation and acetylation (*23-25*). Tau protein is abundant in neurons with a function of stabilizing microtubules. The progressive accumulation of abnormal tau protein may lead to instability of the microtubular structure and the consequent loss of effective intracellular transport, and ultimately, neuronal death (*26,27*).

2.4. *Disequilibrium of calcium homeostasis*

Overload of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is one of the key factors that leads to neuron damage or death (*28*). Ca^{2+} is a major intracellular messenger that mediates many physiological responses of neurons to chemical and electrical stimulation. A regulated rise in $[Ca^{2+}]_i$ could trigger many physiological events, while an unregulated elevation in $[Ca^{2+}]_i$ can alter cell viability or induce cell apoptosis through activating proteases (*i.e.* calpains), reinforcing signals leading to caspase activation, or triggering other catabolic processes mediated by lipases and nucleases (*29*).

2.5. *Free radical oxidative damage*

Much evidence supported that free radical induced oxidative damage may play a role in the pathogenesis of AD (*30,31*). Features of brain, including a high content of readily oxidized fatty acids, high use of oxygen, and low levels of antioxidants, make it especially sensitive to oxidative damage. Both postmortem and living patients with AD demonstrated evidence of oxidative damage in brain tissue. Free radicals may attack and damage lipids, proteins, and DNA, lead to

change in structure and function of these molecules, and consequently result in cellular damage, dysfunction and cell death (*32*). Besides, oxidative stress could also enhance Aβ production, which further induces nerve tissue damage (*33*).

2.6. *Mitochondrial impairments*

Mitochondrial dysfunction has a certain impact on the pathogenesis of AD as indicated by impaired mitochondrial respiration observed in brain, platelets, and fibroblasts of AD patients (*34*). Energy failure, increased oxidative stress, and accumulation of Aβ could be caused by dysfunction of mitochondria, which would damage neurons and could explain many of the biochemical, genetic, and pathological features of sporadic AD (*35*).

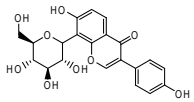
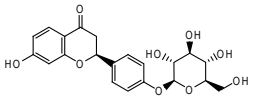
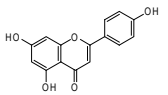
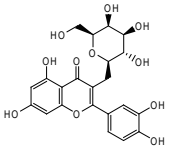
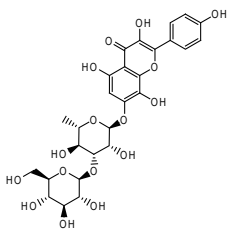
3. Flavonoids as anti-AD agents

Thus far, flavonoids including ginkgo flavonoids, soy isoflavones, puerarin, total flavonoids of Baical Skullcap stem and leaf, liquiritin, apigenin, rhodosin, and hyperoside were reported to have potent effects against AD (Table 1).

3.1. *Ginkgo flavonoids*

Ginkgo flavonoids are the main constituents in the extract of *Ginkgo biloba* (EGB). Ginkgo flavonoids consist mainly of flavonols such as quercetin, kaempferol, and isorhamnetin and biflavonoids like ginkgetin, isoginkgetin, and amentoflavone (*36,37*). These ginkgo flavonoids have free radical scavenging effects and could inhibit lipid peroxidation. Studies demonstrated that mitochondrial DNA from brain of old rats exhibited oxidative damage that is significantly higher than that from young rats (*38*). In addition, mitochondrial glutathione was more oxidized and peroxide formation in mitochondria was higher in old than in young rats (*38*). Treatment with EGB could partially prevent the indices of oxidative damage in brain from old animals (*38*). Other studies demonstrated that ginkgo flavonoids exhibited neuroprotective effects *via* antioxidant activity in brain damaged mice caused by ischemia-reperfusion (*39*). One randomized, double-blind, placebo-controlled, and multicenter clinical trial indicated that EGB was safe and capable of stabilizing and improving the cognitive performance and the social functioning of AD patients for 6 months to 1 year (*40*). Currently, EGB is used in clinics as a medical drug for treatment of AD in China, France, and Germany.

Table 1. Flavonoids isolated from traditional Chinese medicine in treatment of AD

Agents	Structures or contents	Typical origin	Reference
Gingko flavonoids	Mixture: mainly including quercetin, kaempferol, isorhamnetin, and biflavonoids like ginkgetin, isoginkgetin, and amentoflavone	*Ginkgo biloba* L. leaves	*36,37*
Soy isoflavones	Mixture: mainly including daidzin, daidzein, genistin, genistein, and glycitin, glycitein	*Glycine max*	*41,42*
Total flavonoids of Baical Skullcap stem and leaf	Mixture: mainly including scutellarin, baicalin, and chrysin	*Radix puerariae* roots	*64*
Puerarin		*Scutellaria baicalensis* Georgi stems and leaves	*69*
Liquiritin		*Glycyrrhiza uralensis* Fisch. roots	*73*
Apigenin		*Apium graveolens*	*76*
Hyperin		*Hypericum perforatum* L.	*81*
Rhodosin		*Rhodiola rosea*	*83*

3.2. *Soy isoflavones*

Soy isoflavones attracted much interests in recent years due to its estrogen-like effects and role in influencing sex hormone metabolism. The main constituents of isoflavones are demonstrated to be daidzin, daidzein, genistin, genistein, glycitin, and glycitein (*41,42*). It is thought that soy isoflavones intake is a "natural" way to replenish the aging body's declining estrogen levels and thus relieve menopausal symptoms. A previous study demonstrated that postmenopausal women who undertook estrogen-replacement therapy had a significantly lower risk for the onset of AD than women who did not (*43*). These facts suggested the possible benefits of soy isoflavones in AD prevention and treatment.

Mechanisms of anti-AD effects of estrogen lie in the following aspects (*44,45*). *i*) Estrogen reduces the production of Aβ (*46*). Estrogen is capable of regulating the metabolism of APP to enhance the production of soluble APP and decrease the accumulation of Aβ, thus exerting neuroproductive effects. *ii*) Estrogen antagonizes the toxicity of Aβ (*47*). Aβ is capable of promoting lipid peroxidation at the membrane of neuronal cells, leading to production of ROS which further impairs the membrane proteins and breaks the homeostasis of ion balance. The membrane depolarizes and thereby Ca^{2+} influx occurs *via* NMDA receptor channels, which enhances the damage of DNA and lipids and finally leads to neuronal death. Studies indicated that estradiol is a natural anti-oxidant for membrane lipid peroxidation, thereby alleviating the toxicity of Aβ to neurons (*48*). *iii*) Estrogen promotes Ca^{2+} outflow (*49*). Estrogen is capable of releasing intracellular Ca^{2+} *via* non-genomic mechanisms, which is not affected by the concentration of extracellular Ca^{2+}. It was found that estrogen could inhibit the elevation of intracellular Ca^{2+} concentration induced by glutamic acid and antagonize the disequilibrium of calcium homeostasis caused by Aβ. *iv*) Estrogen inhibits inflammation mediated by the transcription factor nuclear factor κB (NF-κB) which is involved in the pathological process of AD (*50*). *v*) Estrogen promotes synaptic growth and expressions of nerve growth factor (NGF) and its receptor (*51*). NGF was demonstrated to be a cytokine that could increase the mRNA levels of choline acetyltransferase, enhance the activities of choline acetyltransferase, and promote the release of acetylcholine. Thus, estrogen is capable of enhancing the effects of NGF. *vi*) Estrogen prevents excessive phosphorylation of tau protein (*52*). Although estrogen exhibits the various above potential actions, its application in clinics for treatment of AD is dismal since it also causes side effects to non-neuronal cells, such as increasing the incidence of breast and endometrial cancer (*53-55*).

Previous studies found that phytoestrogens such as genistein, one of the main ingredients of soy isoflavones, exerted pharmacological effects in a tissue specific manner (*56*). They selectively act on non-reproductive tissues to a certain degree and thus reduce the risk of side effects. Animal studies indicated that soy isoflavones were capable of improving learning and memory abilities through influencing the brain cholinergic system and reducing age-related neuron loss especially in female rats (*57-59*). The underlying mechanisms of favorable effects of soy isoflavones on cognitive function were thought to relate to their potential to mimic the actions and functions of estrogens in the brain (*60*), and promote the synthesis of acetylcholine and neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and NGF in the hippocampus and frontal cortex (*61,62*). A randomized, double-blind, cross-over, and placebo-controlled trial revealed that soy isoflavones were safe and had positive effects on cognitive function, especially verbal memory, in postmenopausal women (*63*). These studies provided evidence of the potential usefulness of soy isoflavones in treatment of AD patients.

3.3. *Puerarin*

Puerarin is an isoflavanone glycoside extracted from species in the family Leguminosae such as *Radix puerariae* and is currently used to treat ischemic cerebrovascular disease and other vascular dysfunctions in China (*64*). Studies found that puerarin had potent effects in improving learning and memory disorders induced by scopolamine or D-galactose in a mouse model (*65*). Yan *et al.* reported that puerarin protected neurons against apoptosis in the cortex and hippocampus of AD rats caused by $Aβ_{25-35}$ through downregulating $Aβ_{1-40}$ and Bax expression in brain tissues, therefore alleviating the spatial learning and memory impairment of diseased animals (*66*). The anti-AD effects of puerarin were also suggested to be related to its abilities in decreasing the lipid peroxidase levels and increasing superoxide dismutase levels in brain tissues, enhancing cerebral blood flow, and improving brain microcirculation (*67,68*).

3.4. *Total flavonoids of Baical Skullcap stem and leaf*

Baical Skullcap is a frequently used traditional Chinese medicine in China. Studies on its active ingredients revealed that the total flavonoids extracted from the stem and leaf, mainly including scutellarin, baicalin, and chrysin, exhibited a series of pharmacological effects such as anti-inflammation, prevention from myocardial damage induced by ischemia-reperfusion, and improved cerebral ischemia (*69,70*). Regarding its effects against AD, Zuo *et al.* found that total flavonoids of Baical Skullcap stem and leaf were capable of protecting hippocampal neurons against damage induced by injection of $Aβ_{25-35}$ in hippocampus in rat (*71*). The underlying mechanisms were related to its actions of decreasing the accumulation of lipid peroxide and proliferation of glial cells induced

by $A\beta_{25-35}$ (*71*). Another study conducted by Ye *et al*. demonstrated that the total flavonoids alleviated memory and learning injury and protected morphological change of hippocampal neurons in AD rats induced by $A\beta_{25-35}$ injection (*72*). These studies suggested the potential efficacies of total flavonoids of Baical Skullcap stem and leaf against AD.

3.5. *Liquiritin*

Liquiritin is an extract from the root of *Glycyrrhiza uralensis* Fisch. (*73*). Yang *et al*. investigated the protective effects of liquiritin on primary cultured rat hippocampal neurons (*74*). They found that pre-treatment with liquirtin for 6 h decreased the elevated levels of intracellular Ca^{2+} concentration and neuron apoptosis caused by $A\beta_{25-35}$. On the other hand, liquirtin is capable of enhancing the effects of nerve growth factor in extending neuraxons (*74*). It is worth noting that liquirtin could also specifically inhibit the activity of acetylcholinesterase and promote the differentiation of neuronal stem cells into cholinergic neurons (*74,75*). The neuroprotective and neurotrophic effects make liquirtin a promising agent against AD.

3.6. *Apigenin*

Apigenin is a flavone usually obtained from *Apium graveolens* (*76*). It is a potent chelating agent that could decrease the metal ions participating in radical reactions and therefore reduce the creation of free radicals (*77*). In addition, apigenin could serve as an anti-oxidant to scavenge free radicals such as oxygen, nitric oxide (NO), and superoxide anion. On the other hand, apigenin possesses estrogen-like effects which are similar to the actions of estradiol (*78*). Due to these biological actions, apigenin was reported to protect human neuroblastoma cells SH-SY5Y against apoptosis induced by oxidative stress *in vitro* (*79*). *In vivo*, apigenin was found to improve the memory and learning disorders of aging mice induced by D-galactose (*80*).

3.7. *Other flavonoids*

Hyperoside is a flavonol isolated from species of *Hypericum* (*81*). In the mouse ischemia-reperfusion injury model, hyperoside was capable of inhibiting lactate dehydrogenase activity decline in brain tissues and obviously improve memory and learning disorders of model mice (*82*). Rhodosin is also a flavonol obtained from the root of Rhodiola rosea (*83*). Rhodosin functions as an anti-oxidant which scavenges free radicals, reduces the content of lipid peroxide, and inhibits degeneration of mitochondria in cerebrum cells and hippocampal pyramidal cells (*68*). Administration of rhodosin was reported to be capable of improving the memory and learning abilities of aging or AD mice (*84*).

4. Conclusion and prospects

AD is a chronic neurodegenerative disease in the central nervous system characterized by progressive memory loss and damage of cognition function. The pathogenesis underlying AD is complicated and not yet well clarified. The currently used medications for treatment of AD are mainly symptom-management drugs. Although they do improve symptoms such as memory disorders and play a key role in treatment of AD at present, these drugs are not capable of reversing the progress of AD. Disease-modifying drugs that aim at root causes of AD are the current research focus and represent the future direction of new drugdevelopment.

In light of the pathogenic complexities of AD, it is probably unlikely that single-target drugs will achieve satisfactory curative effects. The main reasons include the following points. *i*) The onset of this disease involves abnormalities of multiple genes such as *APP*, *PS-1*, *PS-2*, and/or *APOE4*. *ii*) The current targets are multifunctional and strong inhibition or activation of one target may lead to undesired side effects. For example, acetylcholinesterase inhibitors may cause accumulation of peripheral acetylcholine, resulting in peripheral acetylcholine responses such as nausea and vomiting. *iii*) The single-target theory overlooks possible molecular interactions which may constitute cross-talk. Intervention in one of them may not finally affect cell functions or status due to compensatory mechanisms. Given these considerations, development of multiple-target drugs that have both neuroprotective and neurotrophic efficacies are rational strategies in treatment of AD.

Flavonoids reviewed in this article exhibit a series of biological actions against AD including increasing the functions of cholinergic neurons, suppressing typical pathology changes such as neuronal apoptosis, and/or regulating neurotrophy and regeneration relevant mechanisms. These pharmacological effects suggest that more flavonoids may be translated into a new type of anti-AD drugs in the future.

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Review

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Primary gastrointestinal stromal tumors: Current advances in diagnostic biomarkers, prognostic factors and management of its duodenal location

Yuesi Zhong[1], Meihai Deng[1], Bo Liu[2], Cheng Chen[1], Mingliang Li[1], Ruiyun Xu[1,*]

[1] *Department of Hepatobiliary Surgery, Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China;*
[2] *Department of General Surgery, Lingnan Hospital of Sun Yat-Sen University, Guangzhou, China.*

Summary **Gastrointestinal stromal tumors (GIST) constitute 1-3% of all gastrointestinal malignancies and is the most common mesenchymal tumor of the gastrointestinal tract. Although GIST were first described in the literature in the year 1941, important advances of kit mutation and tyrosine kinase inhibitors were not made to understand and manage GIST until the last decade. Here current advances in research of possible cellular origin, diagnostic biomarkers and prognostic factors of primary GIST are reviewed, and the management of primary duodenal GIST is focused on due to its specific location. It is possible that personalized assessment and therapy will turn out to be another milestone for primary GIST.**

Keywords: Gastrointestinal stromal tumor, diagnostic biomarker, prognostic factor, primary duodenal GIST

1. Introduction

Gastrointestinal stromal tumors (GIST) contribute about 1-3% of all gastrointestinal malignancies, and is the most common mesenchymal tumor of the gastrointestinal tract. It can also be seen in the omentum, mesentery, and retroperitoneum (*1-3*). GIST was historically classified as smooth muscle, nerve sheath or autonomic nerve tumors (*4-11*); actually GIST were first described by Golden and Stout (*12*) as a set of mesenchymal tumors arising in the bowel wall in 1941, however, until 1983 when Mazur and Clark (*13*) first introduced the term "stromal tumors" for these mesenchymal tumors, the terminology and understanding of GIST were still in chaos. The second milestone for GIST took place in 1998, when Japanese researchers Hirota and his colleagues (*14*) presented that most GIST possessed CD117 (c-kit) mutations that resulted in full-length KIT proteins with ligand-independent activation, and also discovered that most GIST were positive for CD117. The third milestone for GIST was the development of the tyrosine kinase inhibitor imatinib mesylate for advanced GIST by the end of 2000, and for primary GIST in 2009. In 2007, due to the difference in recurrence-free survival (RFS) between groups of imatinib mesylate and the control, a randomized trial (American College of Surgeons Oncology Group (ACOSOG) Z9001) was prematurely terminated and the result promoted Food and Drug Administration (FDA) approval of adjuvant imatinib for primary GIST (*15,16*) (Figure 1).

As GIST can be divided into primary GIST and metastatic or advanced GIST according to the disease stage, GIST can also be divided into esophageal, gastric, duodenal, small intestine, colon GIST *etc*. when considering the location of origin, the present work will focus on recent features for primary GIST, and special focus will be given to the management of primary duodenal GIST, which sometimes is a big challenge for the surgeon.

2. The incidence and possible cellular origin of primary GIST

2.1. *The estimated incidence of primary GIST*

Regarding that the primary definition of "malignant

**Address correspondence to:*
Dr. Ruiyun Xu, Department of Hepatobiliary Surgery, Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510630, China.
E-mail: zysi@163.com

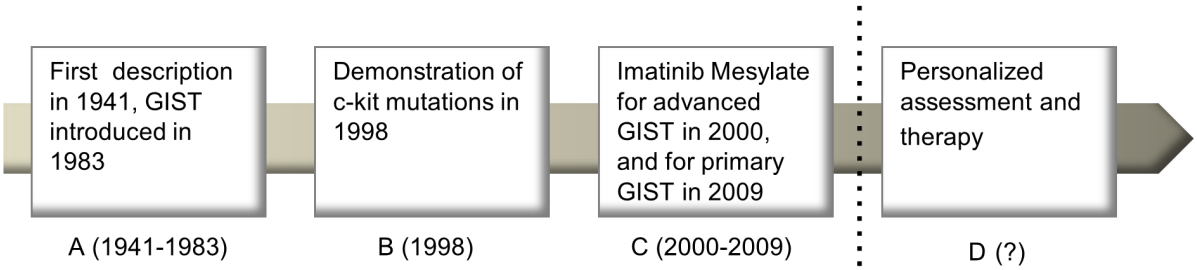

Figure 1. Serial milestones (A-C) of the research history of GIST, and the possible fourth milestone (D) of GIST research in the near future.

GIST" was adopted from criteria in 1990 until it was molecularly assessed in 1998, it was difficult to evaluate the exact epidemiologic data of primary GIST (*17*). The most dependable and exact data of incidence for primary GIST should be from population-based studies that verified all data of all cases of potential GIST (*18-21*), however, there have not been international data for the exact incidence of primary GIST.

According to published data, the estimated incidence of GIST in the United States was approximately 5,000 new cases/year (*8,22*), and that was about 15.91 per million population. The annual incidence of GIST was 13.74 per million population in Taiwan (*21*). In Finland and Sweden the annual incidence of GIST was 10~20 per million population (*1,18*). While the annual incidence of GIST in the Netherlands was 12.7 per million population (*20*) and the incidence of GIST in Iceland was 1.1 per million population (*19*). In HongKong, the annual incidence of GIST before and after the introduction of CD117 was 1.1 per million population and 2.1 per million population respectively (*23*) (Figure 2). The Turkish GIST Working Group (*24*) also reported the national data of 1,160 Turkish cases with a male to female ratio of 1.22 and a mean age of 56.75 years.

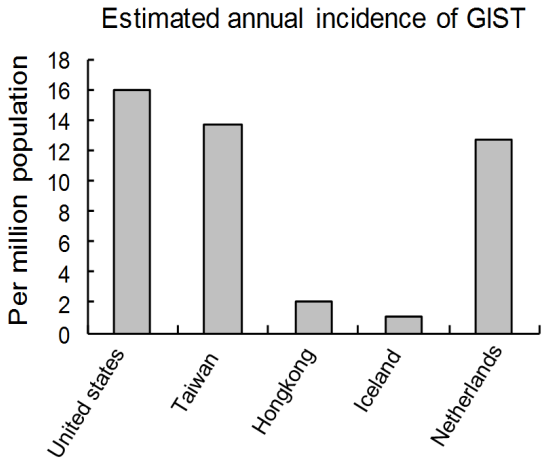

Figure 2. Estimated annual incidence of GIST in different countries and areas according to the literature (*Ref. 1,8,18-24*)**.**

2.2. *The location of primary GIST in the gastrointestinal tract*

GIST contribute 1-3% of all gastrointestinal malignancies, and they are also the most common mesenchymal tumors of the gastrointestinal tract (*1-3*). Although there is a possibility of their ubiquity, it was reported that GIST commonly originated in the stomach (40-60%), small bowel was the second most common site (30-40%), followed by duodenum (about 5%), colon, rectum or appendix (collectively 5-9.3%), and esophagus (2-3%). It was also reported that 12.6% of GIST could arise from omentum-peritoneum, however, GIST were extremely rare in other abdominal locations although they could arise anywhere besides the gastrointestinal tract from the esophagus to the anus (*24-28*).

2.3. *The possible cellular origin of primary GIST*

GIST were historically classified as smooth muscle, nerve sheath or autonomic nerve tumors (*4-11*), and since GIST are the most common mesenchymal tumors of the gastrointestinal tract (*1-3*), there is a trend to conclude that the cellular origin of GIST should be correlated to mesenchymal cells of the gastrointestinal tract. Up until now, is there any exact known origin of primary GIST?

In the year 1995, Huizinga JD *et al*. reported that GIST might be related to the interstitial cells of Cajal (ICC), because lots of gastrointestinal autonomic nerve tumors (a type of GIST before the term GIST were introduced and accepted) were positive for CD34 (*29,30*). Subsequently Kindblom *et al*. and others also proposed that most stromal tumors originate from a mesenchymal stem cell that differentiates toward an ICC phenotype (*31-33*). Actually ICC was first introduced by Cajal SR around 1889, and this type of cell was located in the stroma of the villi, in Auerbach's plexus, deep muscular plexus, circular muscular layer of the intestine, and around the acini and blood vessels of the pancreas, Dogiel subsequently called them ICC. ICC was also known as pacemaker cells and a population of cells in charge of the motility of the gastrointestinal tract (*34*).

In 1998, Kindblom *et al*. (*7*) presented that most GIST were positive for CD117(c-kit) which was first reported by Hirota and colleagues (*14*). Kindblom *et al*. also demonstrated that the ultrastructure and immunophenotype of GIST were similar to that of ICC, which provided stronger evidence for the possible

origin of GIST from ICC or stem cells differentiating to an ICC phenotype. In 2000, Wang Lina *et al*. (*35*) also demonstrated that benign GIST (CD34-negative) were composed of more mature ICC, whereas malignant GIST were composed of dedifferentiated ICC that expressed CD34-positive stem cells.

It seems that the possible cellular origin of primary GIST is ICC or mesenchymal stem cells in the gastrointestinal tract which may subsequently differentiate to a different phenotype and stage of ICC.

3. The diagnostic biomarkers of primary GIST

There is no specified clinical presentation of primary GIST even when the tumor reaches a size larger than 5 cm. When it is a small size, it is asymptomatic; and when the tumor is a large size and symptomatic, the symptoms also vary according to its location and size. The nonspecific symptoms might include abdominal pain, fatigue, dyspepsia, nausea, anorexia, weight loss, fever, obstruction, and chronic or overt GI bleeding. Metastasis can be found 10-15 years post-primary surgery to the lung, bone or liver, however, lymph node metastasis is rare (*36,37*). Sometimes it is very difficult to differentiate GIST from other tumors, such as smooth muscle tumors, schwannomas, desmoid fibromatosis, inflammatory myofibroblastic tumors, inflammatory fibroid polyps, solitary fibrous tumors, synovial sarcomas, follicular dendritic cell sarcomas, glomus tumors, and melanomas (*38*) with clinical presentation and imaging techniques, and then diagnostic biomarkers come to be of huge importance for diagnosis of primary GIST.

Although there is still no serum diagnostic biomarker of primary GIST developed there are some tissue diagnostic biomarkers. As we mentioned before that the second milestone of GIST research is the contribution of Hirota and his colleagues (*14*). They presented that most GIST possessed CD117 mutations that resulted in full-length KIT proteins with ligand-independent activation, they also discovered that most GIST (95%) were positive for the KIT antibody of CD117. This contribution is a breakthough in the diagnosis of GIST. KIT is a member of the type III transmembrane receptor tyrosine kinase family (*39*). 70-80% of GIST possess a *KIT* gene mutation, which were found at exon 11(50-77%), exon 9 (10-18%), exons 13 (1-4%) and exons 17 (1-4%) (*40-46*). All of the above are the first diagnostic biomarkers.

The second diagnostic biomarker is CD34 which is expressed in almost 80% of gastric GIST, 50% of small intestine GIST, and 95% of esophagus and rectum GIST (*47-49*).

The third diagnostic biomarker is the transmembrane protein discovered on GIST1 (DOG1). There are different types of commercial antibodies of DOG1, it is reported that DOG1.1 (Stanford University Medical Center, Stanford, California) and clone K9 DOG1 (Novocastra antibodies, Leica Microsystems, Wetzlar, Germany) were the most applied antibodies. Clone K9 DOG1 was found to be more useful for detecting both KIT-positive and KIT-negative tumors, and tumors with a spindle cell or with an epithelioid morphology. DOG1 is able to detect most CD117-positive GIST and up to 33% of CD117-negative GIST. The sensitivity of DOG1 in detecting GIST varied from 75-100% (*50-52*).

The fourth diagnostic biomarker is a mutation of the platelet-derived growth factor receptor-α (PDGFRA), about 5-7% of GIST present with mutations in the PDGFRA gene in domains similar to those found in the KIT gene. They were mutations in the PDGFRA juxtamembrane domain (encoded by exon 12), the ATP-binding domain (encoded by exon 14) or the activation loop (encoded by exon 18) (*43-45,53*).

The fifth diagnostic biomarker is a combination of the above biomarkers. It was reported that a combination of CD117 and clone K9 DOG1 antibodies can contribute to the diagnosis of 99% of GIST cases (*54*). There will be other combinations of biomarkers for diagnosis of GIST in the future.

4. The prognostic factors of primary GIST

Definitive surgery remains the first option for primary localized GIST that is resectable, and when it came to the third milestone of GIST we presented before, there were gradually adjuvant and neoadjuvant therapy with imatinib or alternative agents for resected and advanced GIST. However, there is an approximately 40-90% recurrence rate after definitive surgery (*54*). Dematteo RP *et al*. (*55*) reported that 127 patients with localized GIST demonstrated a 5-year recurrence-free survival (RFS) rate of 63% after complete resection, they also presented that a tumor size of 10 cm, a mitotic rate of 5/50HPFs, tumor location, and intraperitoneal rupture or bleeding contributed to the postoperative recurrence. Thus prognostic factors become very important for both assessing recurrence risk and the choice of adjuvant and neoadjuvant therapy.

In 2001, The National Institutes of Health (NIH) of the United States convened a GIST Workshop and proposed the NIH consensus classification system (*8*) (Table 1). The NIH classification was based on the lowest level of evidence of consensus opinion, however,

Table 1. NIH classification system for prognosis of primary GIST (2001) (*Ref. 55,56*)

Items	Tumor size in the single largest dimension (cm)	Mitotic count (per 50 HPFs[#])
Very low risk	< 2	< 5
Low	2-5	< 5
Intermediate	< 5	6-10
	5-10	< 5
High	> 5	> 5
	> 10	Any mitotic rate
	Any size	> 10

[#] HPFs: high-power fields.

Table 2. Risk stratification system proposed by Joensuu H. (2008) (*Ref. 56*)

Items	Tumor size in the single largest dimension (cm)	Mitotic count (per 50 HPFs[#])	Primary tumor site
Very low risk	< 2	≤5	Any
Low	2.1-5	≤ 5	Any
Intermediate	2.1-5	> 5	Gstric
	< 5	6-10	Any
	5.1-10	≤ 5	Gstric
High	Any	Any	Tumor rupture
	> 10	Any	Any
	Any	> 10	Any
	> 5	> 5	Any
	2.1-5	> 5	Nongastric
	5.1-10	≤ 5	Nongastric

[#] HPFs: high-power fields.

prognostic factors such as tumor size in the largest dimension and mitotic count included in the system were subsequently proved to be valuable according to the accumulated clinical data evidence. There were four risk categories in the system which were for prognosis rather than for diagnosing whether the tumor was benign or a malignant tumor (*56*).

With the accumulated clinical data evidence, especially data of adjuvant and neoadjuvant therapy with imatinib mesylate, other prognostic factors were proposed besides the NIH consensus classification system. In 2008, Joensuu H proposed a new risk stratification (Table 2) in which tumor rupture and tumor site were added for GIST. The author suggested that the new risk stratification would be useful in selecting adult patients for adjuvant systemic treatments, and identify which patients are most likely to benefit from adjuvant therapy.

Mutation status was also found to be an important prognostic factor for GIST. It was reported that patients with mutation in exon 11 of KIT had a better prognosis when compared to patients with mutation in exon 9 or KIT wild-type when they were treated with imatinib (*57*). Recently, Mazurenko NN (*58*) reported that patients with point mutations and duplication in KIT axon 11 had a better prognosis than those with other KIT mutations, and patients with a PDFGRA mutation had a better prognosis than those with KIT mutations. Watanabe T (*59*) demonstrated that the 2 year recurrence free survival rate of patients who underwent definitive surgery only was lower in patients who had both 557 and 558 codon mutations than those with either 557 or 558 codon mutations.

5. Current advances on management of primary duodenal GIST

Primary duodenal GIST constitute about 5% of GIST in the gastrointestinal tract (*25*), however, primary duodenal GIST contribute 10-30% of all malignant duodenal tumors (*60*). Since definitive surgery is the cornerstone of treatment for primary localized GIST, and lymphnode metastasis is very rare (*61*), segmental duodenal resection with end-to-end anastomosis can be performed on the tumor located in the D1, D3, and D4 segments of the duodenum. However, when the tumor locates in the D2 segment of the duodenum, which sometimes is a big challenge to the surgeon because the D2 segment of the duodenum is a specific location due to the important adjacent anatomical structures (pancreatic head, ampulla of Vater, common bile duct, *etc*.), the surgeon has to decide whether pancreatoduodenectomy or conservative surgery is to be performed for a better outcome. Some researchers argued that conservative surgery was safe and provided similar oncologic outcomes as pancreatoduodenectomy, which should be the choice in patients with GIST in the duodenum that does not involve the pancreatic side of the duodenum (*60*).

In the era of anti tyrosine kinase therapies with imatinib mesylate or other alternative drugs, surgical resection remains to be only considered to be curative, however, duodenal GIST appeared to have the greatest risk of recurrence, when compared to GIST in other locations of the gastrointestinal tract (*62*). It has been confirmed that adjuvant therapy with imatinib mesylate can improve disease-free survival (DFS) and overall survival (OS) of patients (*16,63*). Imatinib mesylate was also recommended to be the neoadjuvant therapy for unresectable tumor, which can decrease the tumor size (*64*).Then there is a new question. Since pancreatoduodenectomy is a complicated procedure with a relatively higher incidence of complications, is it possible for the patient with a large GIST in the D2 segment of the duodenum receive neoadjuvant therapy with imatinib mesylate or other alternative drugs due to imatinib mesylate resistance?

Unfortunately, there are no data of prospective randomized clinical trials focusing on neoadjuvant therapy for large GIST in the D2 segment of the duodenum. Recently in a retrospective multi-center study, Colombo C *et al*. (*65*) reported that neoadjuvant therapy with imatinib mesylate might facilitate surgical resection and increase the chance of preserving normal

biliary and pancreatic anatomy.

6. Prospects for the future

Since 1983 when the term GIST was first introduced, there have been several important advances in GIST research, among which have been discovery of kit mutations and CD117 positive results in GIST as breakthoughs. Recently DeMatteo RP (*66*) proposed a concept of personalized therapy for GIST, we agree with this concept, because there are accumulating research data in biology, such as genetic mutations, and adjuvant or neoadjuvant therapy with systemic medicines, such as tyrosine kinase inhibitors. Personalized assessment and therapy may appear to be the fourth milestone for GIST research (Figure 1). As focusing on primary duodenal GIST, prospective randomized clinical trials are needed for evaluating the outcome of neoadjuvant therapy followed by conservative surgery in patients with a large GIST in the D2 segment of the duodenum.

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Low concentrations of zoledronic acid are better at regulating bone formation and repair

Xiaomeng Yang[1,2], Yanqin Lu[1], Zhiliang Li[3], Yanzhou Wang[4], Fei Zhao[1,2], Jinxiang Han[1,*]

[1] *Shandong Medicinal Biotechnology Center, Key Laboratory for Biotech Drugs of the Ministry of Health, Key Laboratory for Rare Diseases 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;*
[3] *Wuqing People's Hospital, Tianjin, China;*
[4] *Pediatric Orthopedics, Shandong Provincial Hospital, Ji'nan, Shandong, China.*

Summary **The purpose of this study was to investigate optimal concentrations of zoledronic acid (ZA) in terms of their effect on the proliferation, differentiation, and mineralization of primary osteoblasts (OBs) and fibroblasts (FBs). Primary OBs and FBs isolated from patients with clinical osteogenesis imperfecta (OI) and developmental dysplasia of the hip (DDH) were treated *in vitro* with serial concentrations of ZA ranging from 10^{-3} M to 10^{-13} M. An MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) colorimetric assay, flow cytometry, alkaline phosphatase (ALP) determination activity, and alizarin red staining were used to measure the proliferation, differentiation, and mineralization of cells. The MTT assay indicated that high concentrations of ZA may be toxic to cultured cells. No obvious inhibition was observed with a ZA concentration of 10^{-7} M to 10^{-10} M. Proliferation was evident with a ZA concentration below 10^{-11} M ($p < 0.05$). Flow cytometry analysis revealed that cell cycle was arrested at G1/G0 stage with a ZA concentration ranging from 10^{-10} M to 10^{-8} M. ZA did not enhance ALP activity at a concentration of 10^{-8} M or 10^{-10} M. Alizarin red staining indicated the mineralization of primary OBs with a low concentration of ZA (10^{-12} M). In conclusion, this *in vitro* study indicated that ZA-mediated cell proliferation was dose-dependent and that ZA did not inhibit cell proliferation at concentrations below 10^{-8} M. These findings suggest low concentrations of ZA have more of an effect on cell differentiation and mineralization, so low concentrations are better at regulating bone formation and repair.**

Keywords: Osteogenesis imperfecta, proliferation, differentiation, mineralization

1. Introduction

Zoledronic acid (ZA) is the most potent bisphosphonate (BPs) in clinical use and has been used as an anti-resorptive agent to prevent bone resorption in the treatment of metabolic bone diseases like osteoporosis, Paget's disease, osteolytic disease, hypercalcemia of malignancy, and cancer-related osteolytic lesions (*1-3*). ZA regulates the bone balance by inhibiting bone resorptive activity of osteoclasts (OCs), thus effectively reducing bone loss and bone turnover (*4*,*5*). Recent evidence supports the notion that osteoblasts (OBs) could be target cells for ZA and that this action by ZA may in turn contribute to a decrease in OC formation and activity (*6*) but the mechanism for this action is less clear. Osteogenesis imperfecta (OI) is a heterogeneous group of genetic disorders characterized by low bone mass, increased bone fragility, and susceptibility to bone fractures with variable severity. Its main clinical symptoms include increased bone fragility, osteoporosis, susceptibility to fractures, and bone anisotropy, and OI is accompanied by blue sclerae, dentinogenesis imperfecta, hearing loss, excessive joint laxity, and muscle weakness (*7-10*). At present, ZA is the most promising therapy to treat OI, and this is especially true for its intravenous administration. ZA has become the accepted treatment for both adults and children who suffer from OI. Developmental dysplasia

**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

of the hip (DDH) is an abnormal alignment of the femoral head and acetabulum caused by unilateral or bilateral hip instability; DDH is due to genetics, breech birth, swaddling that dislocates the hips, and other factors (*11,12*). The clinical features of DDH differ vastly from those of OI (*e.g.* no osteoporosis), so in the current study DDH served as the control.

The current study used OBs and fibroblasts (FBs) from children with OI and DDH to investigate the effect of ZA on the biological function of OBs and FBs at the cellular level. This was done to determine the optimal concentration of ZA in terms of its effect on the proliferation, differentiation, and mineralization of primary OBs and FBs and to provide a theoretical basis for clinical use of ZA. This study explored the feasibility of ZA because of its role in bone formation and explored its use to treat bone-related diseases.

2. Materials and Methods

2.1. *Materials*

Bone and skin tissue were collected from children with OI and DDH. The study protocol was approved by the Ethics Committee of Shandong Medical Biotechnology Center, Ji'nan, Shandong, China and written informed consent was obtained.

2.2. *Cell lines and cell culture*

Primary OBs and FBs were obtained by collagenase digestion of bone and skin tissue from children with OI and DDH. Tissue was washed and placed in culture dishes with preheated phosphate buffered saline (PBS) (Gibco, NY, USA). Tissue was cultured in 5 mL serum-free Dulbecco's modified eagle medium (DMEM) (Gibco), and 12.5 U/μL collagenase type I (Sigma, NY, USA) was added for digestion at 37°C for 3 h. Cells were centrifuged and resuspended in growth medium. Cells were cultured in DMEM (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) and 1× penicillin-streptomycin (Beyotime, Shanghai, China). Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C in 75 cm^2 plates. Medium was changed every 3 days until cell density reached 90%. Cells were then serially passaged and digested at 37°C for 3 min with 1 mL 0. 25% trypsin (Beyotime).

2.3. *Assay of alkaline phosphatase (ALP) activity (histochemistry)*

Four types of cells were cultured for 9 days in 6-well culture plates at a density of 1×10^4 cells/well. Cells were washed with 4°C precooled PBS (Gibco) and then fixed with 4% paraformaldehyde (PFA) (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) for 10 min before they were washed with PBS (Gibco) (4°C precooling). The samples were then incubated for 30 min with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate (Beyotime) at 37°C. The reaction was terminated by removing the substrate solution and washing with distilled water. ALP-positive cells appeared dark blue.

2.4. *Cell viability assay*

Four types of cells were cultured for 24 h in 96-well culture plates at a density of 2×10^3 cells/well and then growth medium was replaced. Cells were treated with ZA (SELLECK, Houston, USA) at concentrations of 10^{-3} M to 10^{-13} M at various times (2, 4, and 6 days). At the indicated times, a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) assay was performed by incubating cells with 20 μL 5 mg/mL MTT solution/well at 37°C for 4 h. The reaction was then stopped with 150 μL dimethyl sulfoxide (DMSO) (Amresco, OH, USA)/well. Color development was then analyzed by measuring absorbance at 490 nm (A490), and A490 thus corresponded to the viability of cells.

2.5. *Cell cycle analysis (flow cytometry)*

OBs and FBs were cultured in 6-well culture plates at a final density of 6.5×10^4 cells/well with or without 10^{-8} M, 10^{-9} M, or 10^{-10} M ZA for 2 days and 4 days, respectively. After culturing, cells were digested at 37°C for 3 min with 0.3 mL 0.25% trypsin. Cells were collected in 1.5 mL tubes and washed and they were then fixed with 4% PFA (Sinopharm Chemical Reagent Co. Ltd., Beijing, China) for 12 h in 4°C. Cells were washed with precooled PBS and stained with propidium (PI) (Beyotime) at 37°C for 30 min away from light. Flow cytometry was performed as described in the instructions to the Cell Cycle and Apoptosis Analysis Kit (Beyotime).

2.6. *Assay of ALP activity (biochemistry)*

Four types of cells were cultured as previously described in 6-well culture plates at a final density of 6.5×10^4 cells/well. They were treated with ZA for 6 days at a final concentration of 10^{-8} M, 10^{-10} M, and 10^{-8} M with dexamethasone (Sigma-Aldrich) serving as a positive control and growth medium without ZA serving as a normal control. Medium was replaced every 3 days. Samples were washed with PBS (Gibco) and digested and then cells were collected. One percent SDS cell lysis solution was added to each sample to lyse cells at 4°C for 2 h. Cell lysates were then obtained for analysis. The moieties of cell lysates were used to analyze protein content using the BCA Protein Assay Kit (Beyotime), with 5 μL/well cell lysates in 96-well plates. Color development was then analyzed by

measuring the optical density (OD) at 495 nm (OD_{495}). The moieties of cell lysates were analyzed to detect ALP activity by adding 80 μL *p*-nitrophenylphosphate (*p*-NPP) (Sigma-Aldrich) and incubating 40 μL/well cell lysates in 96-well plates at 37°C for 20 min. The reaction was then stopped with 3 N NaOH. The amount of *p*-nitrophenol (*p*-NP) product, corresponding to ALP activity, was measured at 405 nm. Activity was calculated using the formula [ALP activity = A/(X/5 × 40)].

2.7. *Mineralized matrix formation*

OBs from patients with OI were cultured in 24-well culture plates at a density of 8×10^3 cells/well. When the cells reached confluence, the medium was changed to induction medium (10% FBS (Gibco) and 1× penicillin-streptomycin (Beyotime), 500 μg/mL L-ascorbic acid (Sigma-Aldrich), and 10^{-2} mol/L β-glycerophosphate disodium salt hydrate (Sigma-Aldrich) for control cells. For treated cells, the medium was changed to induction medium with ZA at a final concentration of 10^{-6} M, 10^{-8} M, 10^{-10} M, and 10^{-12} M. Medium was replaced every 3 days. Matrix formation was detected at 18 and 21 days by washing cell matrix layers three times with PBS, fixing them with ice-cold 4% PFA (Sinopharm Chemical Reagent Co. Ltd.) for 10 min, and then washing them with distilled water. Matrix layers were then thoroughly stained with 1% alizarin red (Sinopharm Chemical Reagent Co. Ltd.) for 10 min, and excess stain was removed with distilled water. Mineralized matrix formation appeared in red.

2.8. *Statistical analysis*

Results are expressed as the mean ± standard error of the mean (SEM). One-way ANOVA was used to determine the statistical significance of differences between the means of experiments if data had a normal distribution. A Wilcoxon test was used for non-parametric data, and $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. *Assay of ALP activity (histochemistry)*

After plates were stained, microscopic images revealed OBs with a long, fusiform shape, elongation, abundant cytoplasm, and a clear nucleus. Cells grew in clumps and overlapped. FBs also had a long, fusiform shape with a round or elliptical nucleus, and FBs were larger than OBs. All of the four cell types were dyed dark bluish-purple, consistent with the characteristics of OBs and FBs. More OBs were stained and they had a deeper color than FBs. OBs had greater ALP activity than did FBs (Figure 1).

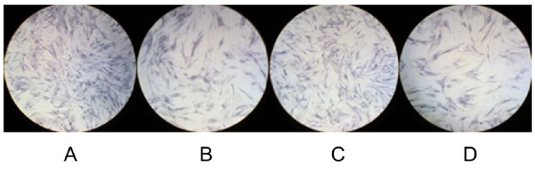

Figure 1. Microscopic images of four types of cells after histochemical staining. (A), OBs from patients with DDH; **(B)**, FBs from patients with DDH; **(C)**, OBs from patients with OI; **(D)**, FBs from patients with OI. All four types of cells were stained deep bluish-purple.

3.2. *Cell viability assay*

Action of ZA was detected at three times (2, 4, and 6 days). ZA at a concentration of 10^{-6} M and higher inhibited the proliferation of OBs from patients with OI and DDH ($p < 0.05$). A ZA concentration between 10^{-7} M to 10^{-10} M slightly inhibited the proliferation of cells. ZA concentrations below 10^{-11} M tended to promote cell proliferation ($p < 0.05$). All of the ZA concentrations tested had little effect on FBs viability at the observed times. In addition, cell proliferation decreased most with ZA concentrations greater than 10^{-6} M (cell viability was approximately 90% of the control), but the rate of inhibition increased over time. There was a dose-dependent change in cell viability with a ZA concentration of 10^{-6} M or lower. Inhibition diminished with a lower ZA concentration as time passed. A ZA concentration below 10^{-11} M tended to promote cell proliferation. Proliferation of cells from patients with OI was promoted more than was the proliferation of cells from patients with DDH (Figure 2). *Note*: cell proliferation rate (%) = (OD of treated cells – OD of control cells)/OD of control cells × 100%.

3.3. *Cell cycle analysis*

Treatment with ZA concentrations of 10^{-8} M, 10^{-9} M, or 10^{-10} M arrested both OBs and FBs from patients with OI and DDH in the G1/G0 phase. Cell cycle arrest was more obvious in OBs than in FBs. ZA had more of an effect after 4 days than it did after 2 days, and it had more of an effect on cells from patients with DDH than it did on cells from patients with OI (Figure 3). *Note*: cell arrest rate = (percentage of ZA-treated cells in G1 phase – percentage of control cells in G1 phase)/percentage of control cells in G1 phase × 100%.

3.4. *Assay of ALP activity (biochemistry)*

ALP activity is a well-known marker of OBs differentiation. After 6 days of culturing, ALP activity was measured. Compared to the positive control (with 10^{-8} M dexamethasone) and normal control, 10^{-8} M and 10^{-10} M had no effect on ALP (Figure 4).

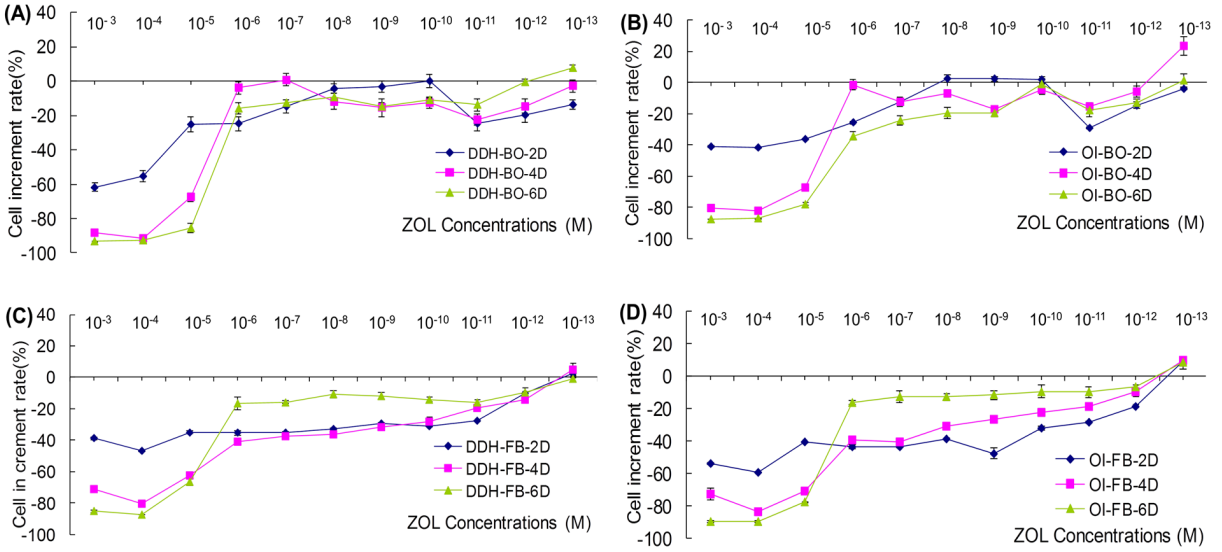

Figure 2. OBs and FBs from patients with OI and DDH cultured in decreasing concentrations of ZA and assessment of the cell count and viability at 2, 4, and 6 days using MTT. (A), Effect on the proliferation of OBs from patients with DDH. At a concentration of 10^{-6} M and higher, ZA inhibited the proliferation of OBs from patients with OI and DDH ($p < 0.05$) after 2, 4, and 6 days of treatment. A ZA concentration between 10^{-7} M to 10^{-10} M slightly inhibited the proliferation of cells. At concentrations below 10^{-11} M, ZA tended to promote cell proliferation ($p < 0.05$), and this trend was more apparent with longer treatment; **(B)**, Effect on the proliferation of OBs from patients with OI (same as in **(A)**); **(C)**, Effect on the proliferation of FBs from patients with DDH. Generally, ZA inhibits FBs. ZA at concentrations greater than 10^{-6} M ($p < 0.05$) resulted in a significant decrease in the cell count compared to untreated control cells as time passed. At concentrations greater than 10^{-6} M, inhibition of cell proliferation diminished as time passed ($p < 0.05$). At concentrations below 10^{-11} M, cell proliferation tended to be promoted ($p < 0.05$); **(D)**, Effect on the proliferation of FBs from patients with OI (same as in **(C)**). Clearly, ZA acted on cells from patients with OI more than it did on cells from patients with DDH.

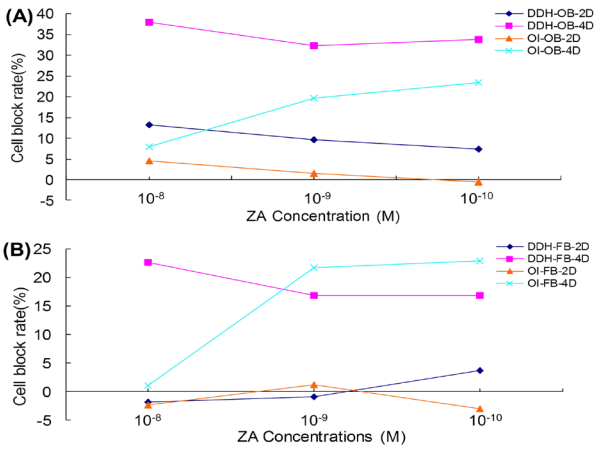

Figure 3. Effect of ZA on cell cycle arrest. (A), ZA arrested the cell cycle of OBs in 2 days. This was true for OBs from patients with OI and those with DDH. After 4 days, the percentage of cells with a cycle arrested at G1/G0 decreased to 38%. A greater percentage of cells from patients with DDH than from patients with OI had their cycle arrested; **(B)**, After 2 days, ZA had no effect on the cell cycle of FBs in the G1/G0 phase. After 4 days, a 10^{-8} M concentration of ZA inhibited cells from patients with DDH at a rate of 23%; this was higher than the rate of inhibition of cells from patients with OI.

3.5. *Mineralized matrix formation*

In the absence of ZA, OBs stained positive with alizarin red in induction medium after 18 days, as did cells treated with 10^{-12} M ZA. A ZA concentration of 10^{-12} M promoted mineralization (Figure 5).

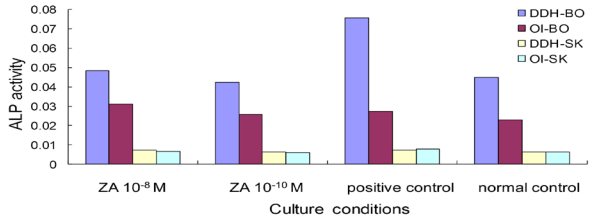

Figure 4. Absence of ZA's effect on ALP activity. Two concentrations of ZA had no effect on ALP, and OBs had greater ALP activity than did FBs.

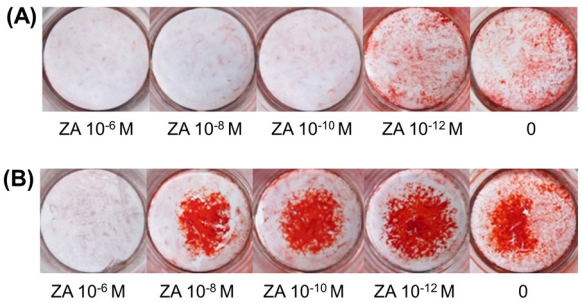

Figure 5. Mineralization staining. (A), After 18 days of culturing, bone nodules were stained with alizarin red. Cultured control cells and cultured cells treated with 10^{-12} M ZA had mineralized matrix deposition in red; staining was more evident in cells treated with 10^{-12} M ZA than in control cells; **(B)**, After 21 days of culturing, all plates had mineralized matrix deposition in red except cells treated with 10^{-6} M ZA. Staining was more evident at a lower ZA concentration, and cells treated with 10^{-12} M ZA had more evident staining than control cells did.

4. Discussion

The first BP was approved in the US in 1977, and BPs are now widely used for the treatment of osteoporosis, hypercalcemia of malignancy, and Paget's disease. Clinical studies have revealed that third-generation BPs have greater efficacy and are more effective at preventing bone-related events. If treatment with other BPs fails, ZA is available. ZA is currently the only BP approved for use in treating a variety of bone diseases. Numerous reports have described the action of BPs on OCs. BPs can have a negative impact on OCs, inhibiting their formation and recruitment (*13,14*), inhibiting the activation of OCs by OBs (*15-18*), inhibiting the maturation and activity of OCs (*19,20*), and promoting the apoptosis of OCs (*21,22*). However, there is no experimental evidence that BPs can reduce the number of mature OCs (*23*). Nitrogen-containing BPs can act by inhibiting the mevalonate pathway and nitrogen-free BPs can act by interfering with the process of energy conversion inside a cell (*24,25*).

Numerous reports have described the role of BPs and their action on OBs in terms of their effect on the proliferation, differentiation, and mineralization of OBs, but the conclusions of these reports differ (*26-29*). The current study was an *in vitro* study. Findings indicated that ZA affected the proliferation of primary OBs in a dose-dependent manner. ZA is also toxic to human OBs at concentrations below 10^{-6} M, since ZA inhibited OBs and FBs proliferation. This inhibition was more evident over time, and the rate of inhibition was about 90% after 6 days of culturing. ZA concentrations from 10^{-7} M to 10^{-10} M slightly inhibited cell proliferation. ZA concentrations greater than 10^{-10} M tended to promote cell proliferation, and this trend was more evident over time. Second-generation BPs can promote the proliferation, differentiation, and mineralization of OBs, and a concentration of 10^{-8} M is optimal (*30,31*). ZA has little effect on cell proliferation (*32*), which is true according to the current results as well. The current results agree with the conclusions of Orriss *et al.* (*33*) and the hypothesis of Maruotti *et al*. (*34*).

ALP is an essential enzyme for bone formation, and ALP is an early indicator to identify and evaluate the degree of differentiation of OBs (*35*). ALP also reflects the activity of OBs. In the current study, treatment with ZA (10^{-8} M and 10^{-10} M) led to changes in ALP activity. Alizarin red staining showed that mineralization/nodule formation increased as the ZA concentration decreased, which is consistent with the findings regarding cell proliferation.

In this study, different concentrations of ZA affected OBs proliferation differently. ZA may affect OI more during mineralization rather than during differentiation. Lower concentrations of ZA may be able to inhibit OCs but also promote OBs and FBs proliferation and differentiation. Selecting an appropriate dose and dosing regimen of ZA may help facilitate bone formation.

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Case Report

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Assessing the value of bilateral inferior petrosal sinus sampling in the diagnosis and treatment of a complex case of Cushing's disease

Changyan Fan[1], Chenran Zhang[2], Xiuhua Shi[3], Liuguan Bian[3], Weiguo Zhao[3], Hua Zhang[4], Tingwei Su[5], Weiqing Wang[5], Xiaoying Li[5], Guang Ning[5], Liang Kong[1], Lingling Hu[1], Qingfang Sun[3,*]

[1] *Department of Neurology, Wuxi No.3 People's Hospital, Wuxi, China;*
[2] *Department of Neurosurgery, Shanghai Institute of Neurosurgery, Shanghai Changzheng Hospital, Shanghai, China;*
[3] *Department of Neurosurgery, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China;*
[4] *Department of Radiology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China;*
[5] *Department of Endocrine and Metabolic Disorders, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China.*

Summary **A 41-year-old female visited Ruijin Hospital because her face was swollen for more than 2 months. The patient was initially diagnosed with Cushing's disease (CD). Several examinations, including a dexamethasone suppression test (DST) at 2 mg and 8 mg, pituitary MRI, abdominal CT, punch biopsy of adrenal masses, and bilateral inferior petrosal sinus sampling (BIPSS), were performed, but the findings were not consistent with the clinical presentation. Ultimately, the patient underwent surgery and recovered. In this case, BIPSS was a useful way to diagnosis CD and suggested the exact location of a pituitary adenoma to Neurosurgery. BIPSS should be a required test for cases of CD that cannot be definitively diagnosed with just an MRI and 8 mg DST before surgery.**

Keywords: Cushing's syndrome, Cushing's disease, bilateral inferior petrosal sinus sampling (BIPSS)

1. Introduction

Cushing's syndrome (CS) results from chronic exposure to excess glucocorticoids produced by the adrenal cortex. CS can be divided into two categories: adrenocorticotropic hormone (ACTH)-dependent (80-85%) and ACTH-independent (15-20%). The former is mostly caused by excess ACTH production and includes *i*) Cushing's disease (CD) typically caused by a pituitary corticotroph adenoma; *ii*) ectopic ACTH syndrome frequently caused by an extrapituitary tumor; *iii*) ectopic CRH syndrome seldom caused by a tumor secreting corticotropin releasing hormone (CRH). The latter results from excess secretion of cortisol by unilateral adrenocortical tumors, either benign or malignant, or by bilateral adrenal hyperplasia or dysplasia (*1-4*).

CD accounts for approximately 70% of cases of CS (*5*). The incidence of CD in the general population is estimated at 0.7-2.4 cases/million/year (*6*), and it affects women 3-5 times more often than men (*7*). Determining the etiology of CS is critical since it allows selection of an appropriate therapeutic regimen because different etiologies have different treatments. Although CD is relatively rare, it most commonly affects adults ages 20 to 50 years. Therefore, an effective treatment resulting in a low rate of recurrence and high curative rate is required (*8,9*). Throughout the literature, however, there are examples of imperfections and pitfalls in all available methods of testing for CD. Hence, the diagnosis of CD is a rigorous process often requiring confirmatory tests at each step and endocrine consultation. Confirmation of the diagnosis of CS and accurate location of its source are vital to optimizing therapy to treat this complex disorder (*10*).

Since venous drainage carrying pituitary-produced ACTH includes the inferior petrosal sinus, sinus sampling is an excellent method by which to distinguish CD from ectopic ACTH syndrome (*11*). If the inferior petrosal sinus (IPS) to peripheral (P) ACTH

Fan CY and Zhang CR contributed equally to this work.

**Address correspondence to:*
Dr. Qingfang Sun, Department of Neurosurgery, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China.
E-mail: rjns123@yahoo.com.cn

ratio (IPS/P) > 2.0 at the baseline and/or ≥ 3.0 after CRH, this is consistent with pituitary-related ACTH overproduction. If IPS/P < 2.0, this corresponds to ectopic ACTH syndrome (*2,12*). This index can be used to differentially diagnose ectopic ACTH syndrome and primary adrenal adenoma. The present study proposes criteria for locating lesions in patients diagnosed with CD at Ruijin Hospital. Per the criteria, a side-to-side gradient ≥ 1.4 indicates a lateral lesion, and a gradient < 1.4 indicates a midline lesion or diffuse hyperplasia. This can help the neurosurgeon more accurately resect the adenoma. BIPSS sensitivity increases with expression of corticotropin-releasing hormone or desmopressin (*13*). In the present study, outcomes were achieved without medication.

2. Case report

Neurosurgery at Ruijin Hospital has successfully treated several cases of CD (*14*). Reported here is a case that was difficult to diagnose.

2.1. *First hospital visit*

A 41-year-old female whose "face has been swollen for more than 2 months" visited Ruijin Hospital for diagnosis and treatment. The patient had central obesity with supraclavicular fat accumulation, a cervical fat pad, thinned skin, no purple striae, high blood pressure, acne, hirsutism, no menstrual irregularity, no pain upon palpation in the area of both kidneys, Tinea unguium infecting both feet, and mild pitting edema on both legs. Abdominal ultrasound revealed bilateral adrenal gland space-occupying lesions with distinct margins and a homogenous internal echo (Figure 1). Thus, the patient was admitted to the Department of Endocrine and Metabolic Disorders at this hospital to screen for CS. Further examination revealed that sex hormone, parathyroid hormone, and growth hormone levels were all normal but serum cortisol (BFC) was elevated and had lost its diurnal rhythmicity (Table 1), 24 h urinary cortisol (UFC) was significantly elevated, and plasma ACTH was normal. Enhanced CT scans of both adrenals revealed the presence of two adrenal adenomas (Figure 2A). MRI suggested a pituitary Rathke's cyst (Figure 3). The patient also had hypertension (150/96 mmHg), osteoporosis, a urinary tract infection, and low potassium; results of a 2 h oral glucose tolerance test did not support a diagnosis of "diabetes".

Results of a dexamethasone suppression test (DST) at 2 mg and 8 mg (Table 2) did not coincide with the patient's clinical symptoms. Since several factors may have affected the DST results, the patient was temporarily discharged to eliminate those factors and the 2 mg and 8 mg DST were repeated.

2.2. *Second hospital visit*

One week later, the patient was readmitted to the Department of Endocrine and Metabolic Disorders at this hospital for a clear determination of etiology. The patient's BFC and 24 h UFC are shown in Table 1 and results of the 2 mg and 8 mg DST are shown in Table 3. Bilateral inferior petrosal sinus sampling (BIPSS) was

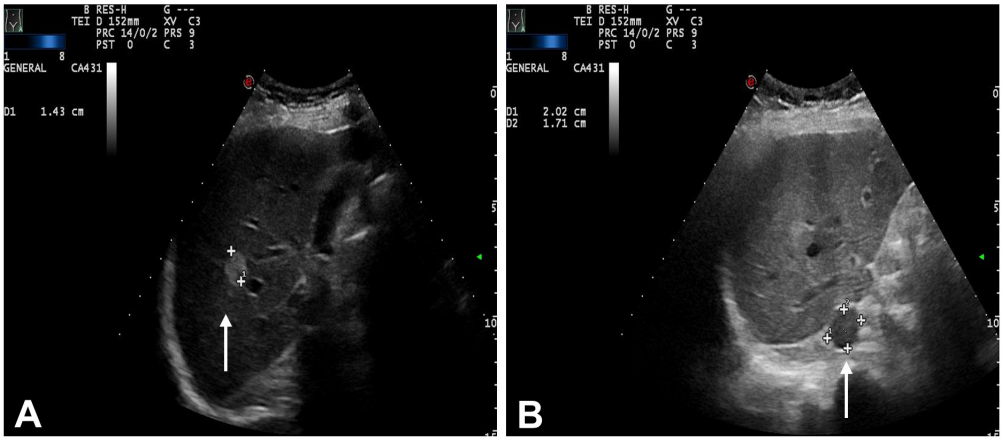

Figure 1. Adrenal ultrasonography: Bilateral adrenal space-occupying lesions. The right adrenal gland had a hypoechoic region about 20 mm × 17 mm in size while the left adrenal gland had two hypoechoic regions about 41 mm × 28 mm in size. The margins were distinct and the internal echo was homogeneous.

Table 1. Endocrine levels prior to transsphenoidal surgery

Items	08:00 BFC (μg/dL)	16:00 BFC (μg/dL)	24:00 BFC (μg/dL)	24 h UFC (μg/24 h)	ACTH (pg/mL)
First visit	40.48	27.52	41.45	2939.30	66.80
Second visit	30.83	39.31	13.64	555.40	38.80
Normal	7.0-22.0	/	/	20.0-90.0	12.0-78.0

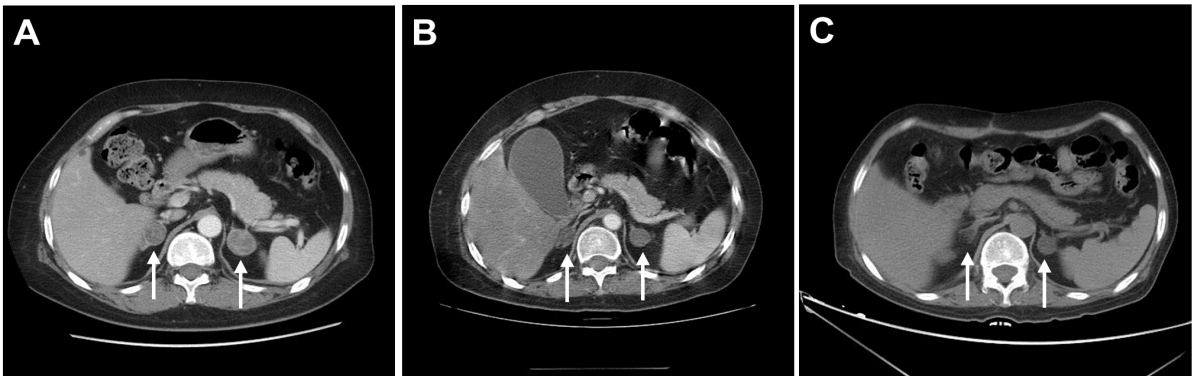

Figure 2. Follow-up of bilateral adrenal lesions with CT. (A), Before TSS, the adrenal adenoma on the left was 41 mm × 28 mm in size and that on the right was 20 mm × 17 mm; **(B)**, Four months after TSS, the adrenal adenoma on the left was 19.7 mm × 17.2 mm in size and that on the right was 17.2 mm × 14.2 mm; **(C)**, Seven months after TSS, the adrenal adenoma on the left was 16.3 mm × 19.1 mm in size and that on the right was 16.7 mm × 14.9 mm. The left adrenal adenoma was significantly smaller but the size of the right adrenal adenoma had changed little.

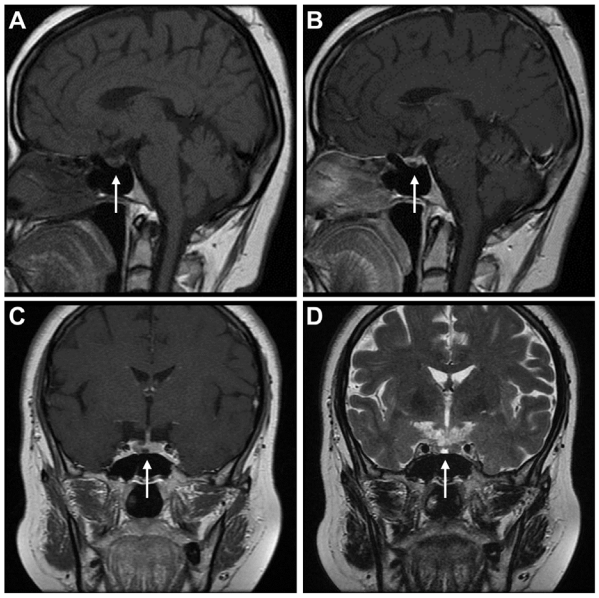

Figure 3. Results of a preoperative pituitary MRI suggesting a pituitary Rathke's cyst (A-D).

Table 2. Dexamethasone suppression test (DST) at 1 mg, 2 mg, and 8 mg during the first visit

DST	BFCd2 (μg/dL)	BFCd3	UFCd2 (μg/24 h)	UFCd3
1 mg	17.29	-	-	-
2 mg	11.50	8.92	90.94	331.65
8 mg	3.64	3.97	21.98	129.30

The baseline level of BFC was 40.48 μg/dL and that of 24 h UFC was 1336.10 μg/24 h

done to provide a definitive diagnosis. And the results of the BIPSS as shown in Table 4.

2.2.1. *BIPSS procedure*

A digital flat-panel angiography system (INNOVA, General Electric Medical System, Milwaukee, WI, USA) was used to perform BIPSS. Bilateral venous catheterization of the IPS was performed by an experienced radiologist at Ruijin Hospital. Following

Table 3. Dexamethasone suppression test (DST) during the second visit

DST	BFCd2	BFCd3	UFCd2	UFCd3
2 mg	7.83	7.93	82.20	7.93
8 mg	2.83	3.41	31.60	242.90

The baseline level of BFC was 30.83 μg/dL and that of 24 h UFC was 555.40 μg/24 h during the second visit.

Table 4. Results of BIPSS

Items	Left	Right	Peripheral
ACTH1 (pg/mL)	82.40	2676.00	66.90
ACTH2 (pg/mL)	82.40	2960.70	72.10

systemic sterile preparation and anticoagulation of both femoral veins at the groin with heparin, the venous sheath was inserted, followed by a guide wire (Terumo, Tokyo, Japan); the needle and wire were replaced with a venous sheath. This was repeated on the opposite side. Catheterization was performed with fluoroscopical guidance using a 5Fr and 4Fr (Terumo) catheter inserted percutaneously into the right and left IPS, respectively. Contrast material (1-2 mL) was carefully injected to obtain digital subtraction venograms of both petrosal and cavernous sinuses to assess the precise location of the catheter tips. During the procedure, catheter positions were verified fluoroscopically. Bilateral central and peripheral blood samples were simultaneously obtained from the sheath at 0 min and 5 min. The catheter was removed and the groin was compressed until venous hemostasis. Blood samples were immediately placed into tubes containing sodium ethylenediamine tetraacetic acid. These tubes were placed on ice and centrifuged at 4°C to determine plasma ACTH levels (Figure 4).

Endocrinology sought a consult from Neurosurgery and Urology since CD had been diagnosed but the characteristics of the adrenal space-occupying lesions and their relationship to clinical manifestations were unclear. Subsequent examination revealed that the patient had a normal erythrocyte sedimentation rate (ESR) and ferroprotein levels and no significant elevation of LDH.

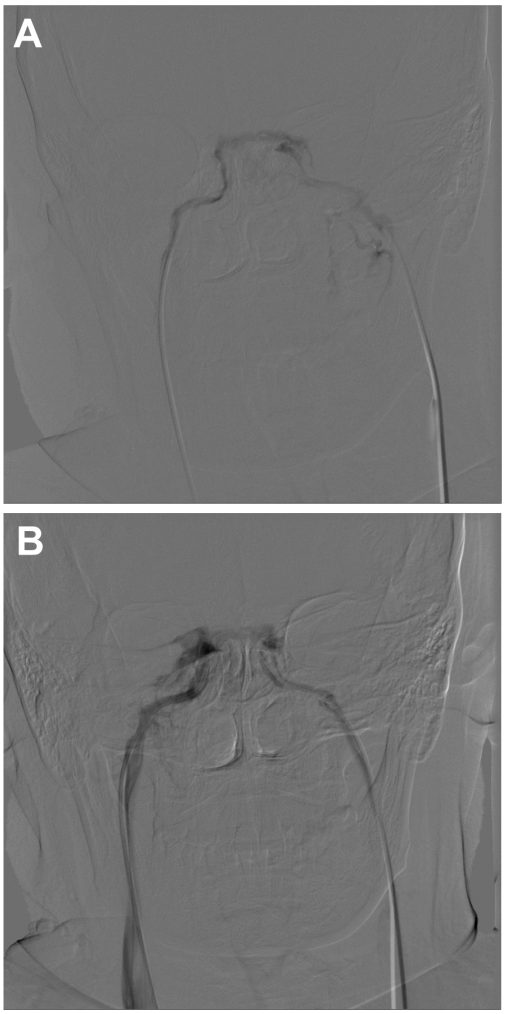

Figure 4. Images from bilateral inferior petrosal sinus sampling. (A), After contrast agent was injected into the left catheter bilateral inferior petrosal sinus, the right inferior petrosal sinus was also visualized; **(B)**, After contrast agent was injected into the right catheter bilateral inferior petrosal sinus, the left inferior petrosal sinus was also visualized.

These did not suggest adrenal tuberculosis, lymphoma, or metastatic lesions. The patient underwent a punch biopsy of the adrenals. Pathology indicated cortical cell hyperplasia in the right adrenal and cytology revealed growth of nests or cords of cells, further suggesting a diagnosis of CD.

Since both inferior petrosal sinus sampling and adrenal biopsy results confirmed CD, Neurosurgery recommended resection of the pituitary adenoma. In October 2010, the pituitary adenoma was removed *via* a transsphenoidal approach under general anesthesia. Perioperative findings were a yellow-white tumor that was solid and that had that had a limited blood supply. The resected area was approximately 1.0 cm × 0.8 cm × 0.8 cm, and the tumor was located slightly off the midline to the right. Pathology indicated a pituitary adenoma (multi-hormone adenoma), and immunohistochemical staining results were ACTH+, FSH+, GH+, PRL+, LH-, TSH-, reticular cell-.

On Day one postoperatively, the BFC and 24 h UFC level decreased significantly (Table 5); sex hormone levels, growth hormone levels, and thyroid function all were normal. Postoperatively, the patient developed diabetes insipidus. Pituitrin was administered, the patient's water-electrolyte balance was maintained, and hormone replacement and symptomatic and supportive therapy were given. The patient's condition was generally satisfactory.

Table 5. Postoperative follow-up of endocrine levels

Items	Baseline	1d-PO[a]	40d-PO	3m-PO[b]	6m-PO[c]
00:80 BFC (μg/dL)	40.48	3.07	4.12	-	7.35
16:00 BFC	27.52	3.05	1.28	-	19.50
24:00 BFC	41.45	5.13	4.77	-	10.56
24h UFC (μg/24 h)	939.30	55.30	271.40	-	74.40
ACTH (pg/mL)	66.80	10.10	17.30	-	8.33

PO, postoperatively; [a,b,c], Hormone therapy with cortisone acetate.

Postoperative follow-up of the BFC and 24 h UFC is shown in Table 5.

2.3. *Third hospital visit*

In May 2011, the patient was admitted to Neurosurgery because of nausea and vomiting lasting one week. The patient's thyroid function and sex hormone levels were all normal. BFC and 24 h UFC were lower than normal, so the patient received hormone replacement therapy. Pituitary MRI revealed postoperative changes in the pituitary adenoma, non-uniform signal intensity in the sella, no significant abnormalities in the remaining brain parenchyma, and no signs of hydrocephalus (Figure 3E-F). Blood electrolytes were Ca 3.47 mmol/L and K 3.23 mmol/L. After infusion of large volumes of fluids and intravenous potassium, electrolytes were Ca 3.00 mmol/L and K 3.33 mmol/L. The patient was transferred to Endocrinology for tests to determine the cause of hypercalcemia. Both times, the patient's parathyroid hormone (PTH) level was lower than normal, inhibiting calcium. Parathyroid ultrasonography revealed no obvious abnormalities. The patient's 24 h urinary calcium level was lower than normal, and renal function results were urea 6.5 mmol/L, creatinine 327.0 μmol/L, and uric acid 837.0 μmol/L. These findings suggested a severe decline in the renal glomerular filtration rate. Hypercalcemia may have been due to the decrease of in the renal glomerular filtration rate. This was remedied with renoprotective therapy and instructions to follow a low-salt diet and drink more water.

The patient had bone pain, and a bone scan suggested lesions of the 4th and 5th lumbar vertebrae and the left sacroiliac joint. Cytology following a bone biopsy revealed no abnormalities, precluding blood diseases. Since the patient complained of nausea and vomiting, hypercalcemia accompanying renal insufficiency was considered. After the patient was given medication to protect the stomach, regulate gastrointestinal function, and supplement potassium, her symptoms gradually

improved. Nausea and vomiting disappeared, renal function gradually improved, and blood calcium decreased. The patient was discharged after her general condition stabilized. After discharge, the patient continued to receive supplements of cortisone acetate and potassium and medication to protect the stomach and kidneys. Four and seven months later, enhanced CT scans of both adrenals revealed the presence of two adrenal adenomas were smaller than before the TSS (Figures 2B and 2C).

3. Discussion

3.1. *The patient*

Preoperative adrenal ultrasonography and a CT examination revealed bilateral adrenal space-occupying lesions about 20 mm × 17 mm in size on the right and about 41 mm × 28 mm in size of the left. Their margins were distinct and their internal echo was homogeneous. The lesions had an intact capsule and homogeneous density, suggesting a larger proportion of fat inside possibly indicating a benign tumor. A correct diagnosis should be reached using a punch biopsy of the adrenals to exclude adrenal hyperplasia, tuberculosis, lymphoma, non-functional adenoma, and other possibilities. The patient underwent a punch biopsy of the right adrenal. Pathology indicated cortical cell hyperplasia and cytology (right adrenal) indicated growth of nests or cords of cells, suggesting CS. This provided a reliable basis for a correct preoperative diagnosis.

Results of both the 2 mg and 8 mg DST did not coincide with clinical manifestations, and enhanced MRI of the pituitary sella revealed no enhanced lesions in the lower part of the anterior pituitary as would suggest a cyst. However, the anterior pituitary was enlarged; in combination with the patient's clinical symptoms and related test results, this suggested pituitary adenoma. Most tests failed to diagnosis this case, but BPSS results suggested that ACTH in the right inferior petrosal sinus was significantly higher than peripheral ACTH (2960.70/72.10 = 41.06, ratio is > 2), and the ratio of ACTH in the right inferior petrosal sinus to that in the left was far greater than 1.4 (2960.70/82.40 = 35.93), supporting a diagnosis of CD and suggesting a tumor partially on the right. After a consult, the patient underwent resection of a pituitary adenoma *via* a transsphenoidal approach. Perioperative findings revealed a tumor located slightly to the right from the midline. Pathology of the pituitary tumor supported diagnosis of an ACTH-secreting adenoma. On Day one postoperatively, BFC, 24 h UFC, and ACTH levels had all decreased. A hormone supplement was provided using cortisone acetate. Postoperative recovery was evident.

Three months postoperatively, the patient failed to receive a follow-up of her hormone levels. She also failed to comply with discharge instructions regarding cortisone acetate. These two factors caused the patient to develop a series of symptoms. Once again, this is a reminder that patients must comply with discharge instructions to avoid unnecessary complications. In this case, BIPSS provided an accurate diagnosis and it also helped to locate the tumor, fully demonstrating its value in diagnosing CD. Although BIPSS is an invasive examination, it is highly sensitive and specific at diagnosing difficult cases of CS. This case report has described a typical case of CS. Thus, use of BIPSS in the diagnosis and treatment of CS should be encouraged in order to improve its diagnostic accuracy.

3.2. *Insights from this case*

Pituitary corticotrophic microadenomas (diameter < 1.0 cm) are responsible for ACTH-dependent CS in most patients. However, identification and precise determination of its location are not always feasible because of the small size. Pituitary MRI and the high-dose dexamethasone suppression test (HDST) have limited value for the differential diagnosis of CS. Moreover, previously used criteria have varied (*15-17*). Despite being invasive and elaborate, BIPSS has been established as a highly accurate diagnostic procedure to distinguish between pituitary and ectopic sources of ACTH. It is specific to the diagnosis and treatment of ACTH-dependent CS. BIPSS has increasingly gained ground among endocrinologists and neurosurgeons (*18-22*).

BIPSS is invasive and some physicians oppose it as a routine examination, stressing that it be limited to the differential diagnosis of CS. In the present case, one radiologist performed BIPSS. Patients usually tolerate the procedure, although some suffer from transient ear, nose, or eye pain or discomfort; their symptoms disappear after the guide wire and catheter are withdrawn. In a previous study of BIPSS by the current authors, only 1 of 52 patients (1.9%) experienced headaches and projectile vomiting; a CT confirmed that contrast agent had leaked into the subarachnoid cavity, but all of the patient's symptoms improved following treatment. The study noted no other serious complications, such as a cerebral vascular accident, groin hematoma, intermittent arrhythmia, or perforation of the right atrium. As long as the operator is careful and gentle, the procedure has a high success rate and low rate of complications (*23,24*).

Accurate diagnosis is crucial to managing the care of patients with CS. Therefore, an ACTH-producing tumor should be accurately and promptly located. Surgical removal of the ACTH-secreting tumor is the primary treatment for patients with ACTH-dependent CS (*25*), and transsphenoidal surgery is the first choice for treatment of CD (*26-28*). BIPSS can assist in diagnosing ACTH-dependent CS and help to locate the tumor during surgery. Therefore, patients should undergo BIPSS if they fail an 8 mg DST or/and the test results are unclear,

pituitary MRI reveals no obvious abnormalities, and endocrine and clinical examinations fail to coincide with results of other tests. In summary, patients who are eligible for BIPSS should undergo the procedure to confirm a diagnosis of CD.

Acknowledgements

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Commentary

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Research on economy and social exclusion: China dolls and rare diseases

Akihiko Matsui*

Faculty of Economics, The University of Tokyo, Tokyo, Japan.

Summary **The second workshop on "Research on Economy And Social Exclusion (REASE)" was held in the University of Tokyo on January 26, 2013. Focusing on rare diseases and disorders in China, three speakers from China introduced the current status of rare diseases and the challenge of support organizations for patients with rare disease and disorders in China, and especially pointed out some important issues associated with rare diseases and disorders in China. From the viewpoint of economics, this paper discusses some of the important issues of rare diseases and disorders in China raised in this workshop, especially from the aspects of economy of scale and orphan drugs, and the emergence of stigma from discrimination. It was shown that international coordination and cooperation are called for in order to give a proper incentive to the drug industries to create new drugs for rare diseases, and suggested that an important step toward inclusion is to reduce stigma by making rare diseases visible as much as possible.**

Keywords: Disability, complementarity, discrimination, prejudice

1. Introduction

On January 26, 2013, the second workshop on "Research on Economy And Social Exclusion (REASE)" was held in the University of Tokyo. In this workshop, focusing on rare diseases and disorders in China, we invited three speakers from China, Rufang Huang, Lei Xiao, and Yitong Jiang. Huang and Xiao are the core members of China-Dolls Center for Rare Disorders (*http://www.chinadolls.org.cn*), which is a non-government organization that assists people with rare diseases and disorders, with particular strength in assisting children suffering from osteogenesis imperfecta (OI) and osteomalacia. They introduced the current status of rare diseases and the challenge of support organizations for patients with rare disease and disorders in China, and especially pointed out some important issues associated with rare diseases and disorders in China, including a lack of specialized medical doctors, a lack of medicine and huge cost, an insufficient social security system, poverty due to these factors, and discrimination and prejudice against patients and their families.

These issues may be regarded as social problems as well as medical ones. Some of the issues cannot be understood and resolved without understanding their economic aspects. This is precisely the reason that social sciences including economics have to be involved in the research of intractable and rare diseases, and this is why it is related to a project called "Research on Economy And Social Exclusion (REASE)" launched by our group. REASE is a research project funded by the grants-in-aid for scientific research, focusing on the economic aspects of barriers and obstacles of people with long-standing health problem or disability, children who need social care, and those who suffer from the east Japan earthquake in 2011.

As pointed out by Tang and Makuuchi (*1*), it is estimated, that there are 5,000-7,000 distinct rare diseases in the world. While each disease is small in terms of the number of patients (0.065-0.1%) as defined by the World Health Organization (WHO), the total number of patients suffering from rare diseases amounts to 6-8% of the total EU population and 10% of the total US population. Therefore, rare diseases have a significant impact on our society.

From the viewpoint of economics, I want to discuss some of the above concerns raised by Rufang Huang, especially from the aspects of economy of scale and

**Address correspondence to:*
Dr. Akihiko Matsui, Faculty of Economics, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: amatsui@e.u-tokyo.ac.jp

orphan drugs, and the emergence of stigma from discrimination.

2. Economy of scale and orphan drugs

The first two issues raised by Rufang Huang, the lack of medical doctors and that of medicines for rare diseases, are understood by using economy of scale, a classical concept of economics (see, *e.g.*, a textbook by Mankiw (*2*)). Although there are a couple of versions of the concept, let us focus on the most relevant one here, that is, a decreasing average cost of production. Let us, for the sake of simplicity, take the case of drugs as an example instead of that of medical doctors.

In order to create a drug, a drug company needs an investment. Ignore uncertainty for the moment and suppose that this research and development (R&D) investment costs $F > 0$. The drug, after development for use, costs $c > 0$ per person. Suppose further that there are n patients potentially using this drug. Then the price p of the drug has to be at least (*i*) $\bar{p} = c + F/n$ in order for the company to break even. The less the number of patients is, the higher the break even price $\bar{p}$ becomes. In the presence of economy of scale, market may not function well. To see this point, suppose that the demand curve for this drug is (*ii*) $d = n - ap$, $a > 0$ where d is the demand as counted by the number of patients. The property that the demand decreases as p increases reflects the fact that the higher the price becomes, the more patients have to give it up. Assume $n/a > c$ for relevancy. If the price is $p < n/a$, then the demand is $n\text{-}ap$, and the consumer surplus, the surplus that the patients get in total, is (*iii*) $(1/2)(n/a\text{-}p)(n\text{-}ap)$. Thus, from the viewpoint of total welfare, which is obtained by subtracting the cost from the sum of the consumer surplus and the company's profit, the drug has to be developed if (*iv*) $(1/2)(n/a\text{-}p)(n\text{-}ap)+p(n\text{-}ap)\text{-}c(n\text{-}ap)\text{-}F > 0$ holds. On the other hand, this drug is provided if and only if the company can raise profit, *i.e.*, (*v*) $p(n\text{-}ap)\text{-}c(n\text{-}ap)\text{-}F > 0$ holds for some $p > c$. A simple calculation shows that this drug is not provided despite that its provision would increase the total welfare if (*vi*) $(p\text{-}c)(n\text{-}ap) < F < (1/2)(n/a\text{-}p)(n\text{-}ap)+(p\text{-}c)(n\text{-}ap)$ holds. In this case, some subsidy is called for in order to properly provide the drug.

Now, to highlight the issue of drug demand in the context of world economy, suppose that there are K countries. Assume that the kth country has n_k patients of this disease. For the sake of simplicity, each country has two strategies, to approve the drug at a small but positive cost if developed, which is denoted by A, and not to approve it, denoted by N. Assume that $n = n_1 + ... + n_K$ is sufficiently large so that (*v*) holds. The benefit from taking A depends on the number of countries that take A: the more countries approve the drug, the higher the benefit from A becomes. Suppose that there is a threshold $\bar{k}$, the number of countries, beyond which taking A induces a higher payoff than taking N. This property that the more countries take A, the more attractive action A becomes is called strategic complementarity in economics (Bulow *et al*. (*3*)).

In this game, there are two types of equilibria. To begin with, let us see the incentive of each country. There are two important cases. First, each country has an incentive to approve the drug if all the other countries are expected to approve it. Second, no country has an incentive to approve the drug if no other country is expected to approve it. Given the behavior of the countries, there are essentially two possibilities of the company's investment decision. On one hand, if the company expects the first case to occur, then it has an incentive to invest in R&D. On the other hand, if it expects the second case, then it has no incentive to invest in R&D. Hence, there are two qualitatively different equilibria, the one in which the drug is developed and provided and the other in which it's not. Note that this is true even if (*v*) holds so that it is beneficial for the entire world to have the drug.

Thus, we need international coordination and cooperation to obtain the second type of equilibrium where the needs of people with rare diseases are properly accommodated.

3. The emergence of stigma from discrimination

In his presentation, Lei Xiao showed pictures of a boy with OI who is denied entrance to an elementary school because the school said they cannot be responsible for a possible broken bone of the boy. This way, OI patients, and people with rare diseases/disorders become invisible in the society. This section, taken from Matsui (*4*), argues that such a discriminatory action may lead to prejudice or stigma.

While stigma has been a key concept in sociology since Goffman (*5*), it has never been a key concept in economics. One reason for this is that stigma is a mental attachment, and there has been little attempt to relate it to economic variables (*Note*: one exception is Becker (*6*), but his analysis assumes stigma at the outset, while the purpose of our analysis is to endogenize it). Kaneko and Matsui (*7*) studied stigma in a game theoretic context, constructing a two-stage game called the festival game. In the first stage of this game, a population, which is divided into two ethnic groups, A and B, simultaneously choose a location, 1 or 2, to visit. Let group A be the majority and group B be the minority. In the second stage, upon observing the ethnic composition of the participants at one's own location, each person decides whether he/she will play in a friendly or an unfriendly manner. If a person takes unfriendly action, then his/her level of satisfaction (payoff, henceforth) is at the default level of zero. On the other hand, if the person takes friendly action, then – since this is a "festival" – his/her payoff depends upon the number of friendly people in the same location. The greater the number of friendly people, the higher the

payoff to the person who takes friendly action. In other words, the festival game exhibits complementarity. If no other people take friendly action, the payoff from taking friendly action is less than that from taking unfriendly action. Here, we assume that even the smaller ethnic group is so large that the group by itself can reach a critical mass beyond which people taking friendly action receive a positive payoff. In order to obtain a clear result, it is assumed that their payoffs do not depend, among other things, upon the demographic composition.

Kaneko and Matsui decomposed the analysis of this game into two parts, the standard equilibrium analysis and a new analysis, called inductive game theory. First, the simplest equilibrium is the one in which everyone goes to the same location and takes friendly action. This is a unification equilibrium. Another simple equilibrium is the one in which people choose a location randomly, and wherever they may go, they take unfriendly action. These are equilibria since people would like to take friendly action if many others do, and vice versa.

Yet, there is another equilibrium, which may be called a segregation equilibrium. In this equilibrium, the two groups of people go to different locations: group *A* people go to, say, location 1, while group *B* people go to location 2. They take friendly action as long as they observe only people from their own ethnic group. In order for this situation to be an equilibrium, each individual in group *B* must have no incentive to deviate to location 1, which is physically more attractive than location 2 since more people gather there and a higher payoff is obtained there than at location 2. This is made possible if group *A* people discriminate against group *B* people. Technically, this can be done if when group *A* people see a group *B* person they suddenly take unfriendly action. This way, segregation is maintained through discrimination.

Kaneko and Matsui continued on to the development of inductive game theory. In this theory, people try to "explain" their experiences by constructing a model. Suppose, for this purpose, that people do not know the actual structure of the game, or in particular, how their payoffs are determined. Suppose further that they play the game according to the segregation equilibrium described above.

In this equilibrium, people who wish to "explain" the discriminatory behavior may come up with the following story. For some reason, group *A* people are happy in general, but they become unhappy from time to time. When one closely monitors what happens when their payoff drops, one may realize that a decrease in payoff is observed whenever there is a group *B* person in location 1. Thus, this group *A* observer may conclude that group *A* people become unhappy when a group *B* person joins them. This is a false model since the objective game says that what matters is the number of friendly people. However, this prejudicial model may well explain one's experiences, and prejudices emerge.

How can such a prejudicial model be falsified and thus an inclusive mind be cultivated? The above prejudicial model would be falsified if two groups interact in a friendly manner, which is perhaps the easiest way of eliminating prejudices in the mind of the people in the main stream.

4. Conclusion

We have shown, by way of economics/game theory, that in an industry for orphan drugs, economy of scale induces market failure in a single country and coordination failure in an international economy, and therefore, international coordination and cooperation are called for in order to give proper incentive to the drug industries to create new drugs for rare diseases. Another concern related to rare diseases is discrimination and prejudice. We have shown that invisibility caused by discrimination enhances prejudice. It is, therefore, an important step toward inclusion is to make rare diseases visible as much as possible.

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Letter

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Revision of measures to combat intractable diseases in Japan: Three pillars will play an even greater role in the future

Peipei Song, Norihiro Kokudo*

Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.

Keywords: Rare diseases, orphan drugs

Summary: *Over the past 40 years, measures to combat intractable diseases in Japan have progressed substantially since the implementation of the "Outline of Measures to Combat Intractable Diseases" in 1972. However, many challenges remain. In order to further promote measures to combat intractable diseases, a "Revision of Measures to Combat Intractable Diseases" was approved by the Japanese Ministry of Health, Labor, and Welfare (MHLW) on January 25, 2013. The revision rests on the three pillars of development of effective strategies to treat intractable diseases and improved care for those affected, creation of fair and consistent mechanisms to reimburse medical expenses, and implementing measures to enhance public understanding and encourage the social participation of those affected. These pillars will play an even greater role in future measures to combat intractable diseases.*

On January 25, 2013, a "Revision of Measures to Combat Intractable Diseases" was approved by the Japanese Ministry of Health, Labor, and Welfare (MHLW) (*1*) to further promote future measures to combat intractable diseases.

Intractable diseases (Nanbyo), usually referred to as "rare and intractable diseases" in Japan, are currently defined as"a disease of unknown etiology with no effective treatment that presents a major financial and psychological burden and that is rare (fewer than 50,000 total patients)" (*2*). The "Outline of Measures to Combat Intractable Diseases" was implemented in 1972. Since then, measures to combat intractable diseases in Japan have progressed substantially, including the "Specified Disease Treatment Research Program" established in 1972 with the support of the MHLW to promote research on 130 intractable diseases, the revised "Orphan Drug Regulation" enacted in 1993 to encourage discovery and development of orphan drugs with specific incentives, the "Intractable Disease Information Center" established in 1997 to provide vast information to patients with intractable diseases, and designation of "Bases for Early and Exploratory Clinical Trials in Specific Research Areas" starting in 2011 to promote the development of innovative drugs and medical devices originating from Japan (*2-4*). However, many challenges remain, such as the varied needs of patients with intractable diseases and their families, gaps in knowledge of tools and strategies to treat intractable diseases, and reimbursement of the considerable financial burden caused by intractable diseases. Given this situation, a "Revision of Measures to Combat Intractable Diseases" was approved in January 2013 in Japan in order to promote measures to combat intractable diseases in light of changing social and financial resources.

The revision clearly described the current state of intractable diseases in Japan and it identified research programs and future strategies to treat intractable diseases. Revision of the measures to combat intractable diseases rests on three pillars. First is the development of effective strategies to treat intractable diseases and improved care for those affected. It includes four important aspects: government-funded special biomedical research programs to enhance basic and applied research on intractable diseases, establishment of a national intractable diseases registry and effective use of databases at the national and international levels, formulation of guidelines and establishment of a support network for specific intractable diseases, and designation of certain hospitals as bases for treatment of intractable diseases. The second pillar is creation of fair and consistent mechanisms to reimburse medical expenses for intractable diseases. Aspects of this pillar include: collection of patient data and establishment of criteria

**Address correspondence to:*
Dr. Norihiro Kokudo, Department of Surgery, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: KOKUDO-2SU@h.u-tokyo.ac.jp

defining a specific intractable disease, diagnosis and certification of individuals with intractable diseases, and determination of appropriate levels of benefits. The third pillar is implementing measures to enhance public understanding and encourage the social participation of those affected by intractable diseases. Aspects of this pillar include: educational campaigns on intractable diseases as well as the provision of social welfare services, employment assistance, and counseling to patients' and their families.

Intractable diseases are a pressing public health issue and a challenge to medical care worldwide. The "Revision of Measures to Combat Intractable Diseases" represents a positive perspective on the development of measures to combat intractable diseases in Japan, and this step may serve as a reference for other countries coping with intractable diseases elsewhere around the world.

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