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

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IRCA-BSSA Group Journals

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

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

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

Copyright
Current progress in the management of rare diseases and orphan drugs in China

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Summary
Currently, the issues of how to treat rare diseases and to improve accessibility to orphan drugs are arousing more and more concerns in China. Here we describe the push and pull incentive policies for rare diseases and orphan drugs and analyze the coverage and reimbursement level of rare diseases in the current Chinese medical insurance system. Three key obstacle factors that hinder Chinese patients’ accessibility to timely drug treatment are summarized. Based on a comprehensive analysis, the measures of orphan drugs legislation, incentive mechanism, supply mechanism, and reimbursement mechanism are urgently expected to be established with the purpose of improving healthcare for patients with rare diseases in China.

Keywords: Rare diseases, orphan drugs, accessibility, management strategies

1. Introduction

In recent decades, there were a series of events concerning the shortage of drug medication for patients suffering from rare diseases reported by the mass media of China. These drugs are always in the literature termed orphan drugs, which most frequently include Clostridium botulinum antitoxin type A, alglucerase injection (Ceredase®), imiglucerase (Cerezyme®), ceramide trihexosidase/α-galactosidase A (Fabrazyme®), zinc acetate, coagulation factor VIII, coagulation factor IX, iron(III)-hexacyanoferrate(II) (Radiogardase®), busulfan, etc. It is also known now that various types of rare diseases exist in China, e.g. osteogenesis imperfecta, neuromuscular diseases, Wilson's disease, Fabry's disease, Gaucher's disease, phenylketonuria, acromegaly, hemophilia A and B, hereditary angioedema, chronic myeloid leukemia, Pompeii disease, mucopolysaccharidosis, lymph-angioleiomyomatosis, albinism, growth hormone deficiency, pulmonary arterial hypertension, botulism caused by type A C. botulinum, and internal contamination with thallium.

Currently, rare disease is defined as an incidence rate of less than 0.65‰ or 1‰ of the disease or symptoms by the World Health Organization (WHO). In terms of WHO's definition of rare diseases, at least 10 million people are suffering from rare diseases in China, given a population of at least 1.3 billion (1). Unfortunately, most of the Chinese patients with such diseases are bearing a deadly physical, psychological, and economic burden due to lack of proper health care and supportive policy or health care insurance. In addition, shortage of orphan drugs has also become a major problem.

Management of rare diseases and orphan drugs has attracted wide attention in recent years in China. Patients and patients' families, patients' advocacy groups, health care professionals, pharmaceutical policy scholars, lawyers, and representatives of The National People's Congress are advocating establishing some protective measures for rare disease, such as rare diseases prevention and treatment law, medical insurance system of rare diseases or medical assistance system.
for rare diseases. In recent times, many changes that favor patients with rare diseases are emerging in China as compared to previously. In our study, we describe three key aspects including incentive policies, medical insurance policies and social supportive activities, with the purpose of exploring current progress in management of rare diseases and orphan drugs in China.

2. Incentive policies for rare diseases and orphan drugs in China

For incentive strategies for global health research and development, funders and advocates have considered solutions of two types: "push" mechanisms and "pull" mechanisms (2). "Push" mechanisms aim to reduce the developer's risks and initial costs, thereby lowering barriers to entry and increasing investment in research at the start of the innovation pathway. In contrast to push mechanisms, "pull" mechanisms are designed to create or secure a market, thereby improving the likelihood of a return on investments. They motivate investment by guaranteeing a reward for the product after completion of its development phase (3). Push mechanisms subsidize research inputs, while pull mechanisms reward research output.

Push mechanisms can take many forms, such as public innovation funding/grants in basic research, subsidies for research, tax credits on research and development, product development partnerships (public-private partnerships), expedited drug regulatory review, facilitation mechanisms, liability protection, and so on. Pull incentive measures include market guarantees, purchase funds, prizes for successful research, improved market information, tax credits on sales, intellectual property incentives, and patent buyouts. For example, advance purchase commitment to buy a new drug, extension of patent term or market exclusivity on a new drug, or transferable patent extension of an alternative drug (2,3).

The orphan drug policies in the United States of America (USA), the European Union (EU) and Japan use the push and pull incentive strategies. The most common push strategies included in the existing orphan drug programs are: research grants, protocol assistance for clinical trials (automatic or on request), fast-track procedures (or high priority), tax credits (mainly for clinical research expenses), and exemption from drug registration fees. In this respect, the Orphan Drug Act in the USA offers particularly favorable conditions with tax credits that can reach up to 50% of clinical costs. Pull strategies in the existing orphan drug programs from the USA and EU have a long market exclusivity for orphan drugs and authorization criteria for orphan drug designation according to clinically superior values. It means orphan drug programs protect the first drug sponsor's benefits (4).

In China, on the one hand, push stimulating policies for orphan drugs allow reduction of sample size of clinical trials and entrance into fast-track procedures. But currently Chinese government does not design special funding/grants or subsidies or priority grants for rare diseases or orphan drug research. On the other hand, the pull stimulating policies for rare diseases and orphan drugs include administrative market protection for traditional Chinese medicine preparations, and an information management system for hemophilia (as a form of improved market information). There are further details as follows.

2.1. Incentive push policies

2.1.1. Provisions for drug registration

This regulation was implemented originally on May 1, 1999, which was called the Chinese New Drug Registration Regulation. Article 27 in the old edition stipulates that if a new drug which could have an effective clinical therapeutic value for life-threatening or difficult critical illness (such as AIDS, cancer, rare diseases, etc.), and was made as the first domestic application, then the review process should be sped up. The latest edition of this provision was carried out on October 1, 2007, and named Provisions for Drug Registration (5). In the latest edition, Article 32 of the provision indicates that the clinical sample size of rare or special diseases can be reduced or can get a clinical trial exemption. Article 45 regulated that State Food and Drug Administration of China (SFDA) may implement special review and approval in case of the following applications: new drugs with significant clinical advantage for the treatment of diseases such as AIDS, malignant tumors, and rare diseases, etc. For drugs specified in the above-mentioned causes, applicants may apply for special review and approval in the process of drug registration. The Center for Drug Evaluation of SFDA shall organize expert meetings to discuss and determine whether or not to conduct special review and approval for the drugs. In 2010, SFDA approved the import of ambrisentan tablets (Volibris®) as an orphan drug and conditionally approved imatinib mesylate tablets (Glivec®) for the treatment of dermatofibrosarcoma protuberans sarcoma (DFSP). The soluble guanylate cyclase (SGC) agonist and long-term non-prostaglandin class of prostacyclin receptor (IP receptor) agonist were also approved to enter clinical trials by SFDA (6).

2.1.2. Requirements for special approval of new drug registration

This requirement was formally issued and implemented as of January 7, 2009 (7). Article 2 points out that new drugs for the treatment of diseases such as AIDS, malignant tumors, and rare diseases, etc. with a significant clinical advantage may apply to enter the
special review and approval procedure in the stage of marketing approval, excluding the stage of clinical trial approval. The requirements follow the general principles for special approval of new drug registration, namely "early intervention, priority review, multi-channel communication, and dynamic data supplement". When a new drug enters the special approval procedure, the marketing approval time of a new drug is about 120 work days. Generally, the standard marketing approval time of a new drug is at least 150 work days. At the same time, the sponsor of the new drug with special approval has a chance to attend the communication meeting and gain protocol technical assistance from the Center for Drug Evaluation of SFDA. In 2010, tinib hydrochloride tablets (Conmana™) as an original new drug in China got special approval. Conmana treats advanced non-small cell lung cancer (6).

2.1.3. Provisions for in vitro diagnostic reagents registration

This Provision of in vitro diagnostic reagents registration was run on July 1, 2007 by the Chinese SFDA (8). Article 31 points out that new in vitro diagnostic reagents for rare diseases and special diseases or other conditions may be allowed to reduce the number of cases required for clinical trials or be exempt from clinical trials, but the applicant needs to submit a registration dossier to apply for a waiver of clinical trials, in which detailed reasons should be provided. Besides, in January 2012, the 2011-2015 plan of national drug safety from the State Council of China clearly indicates encouragement for research and development of orphan drugs (9).

2.2. Incentive pull policies

2.2.1. A protection system for certain traditional Chinese medicine preparations

The State Council of China issued regulations for protection for certain traditional Chinese medicine preparations on October 14, 1992 (10). Thereafter, SFDA released the protection guidelines for certain traditional Chinese medicine preparations in 2009 (11). Article 2 in the protection guideline emphasizes that if a traditional Chinese medicine preparation can make a significant improvement on the critical end point of clinical outcomes (mortality, disability, etc.) for severe or rare diseases (such as phenylketonuria, thalassemia, etc.), it may apply for first-level national protection. A traditional Chinese medicine preparation which gained first-level protection can get a duration of administrative market protection for thirty years, twenty years, or ten years respectively, based on different situations. During the period of protection, the other pharmaceutical companies which also have the same medicine as that of the traditional Chinese medicine protected will be required to stop producing a similar pharmaceutical product, otherwise the market approval of the medicine of any other pharmaceutical company shall be suspended by SFDA.

2.2.2. An information management system of hemophilia cases in China

It is estimated that China has about 60,000-130,000 hemophilia patients, who are in great need of coagulation factor VIII. The Ministry of Health of China released a bulletin board on the establishment of an information management system of hemophilia cases on November 17, 2009. This notice requires that a medical institution in every province should be designated as a provincial information management center of hemophilia patients, which is responsible for collecting and reporting the information of patients with hemophilia including the basic conditions of patient, disease detection, diagnosis, the supply and demand situation of coagulation factors, and other information. Currently, Ministry of Health designated the Department of Blood Diseases Hospital of Chinese Academy of Medical Sciences as a national information management center for hemophilia patients and also announced 31 hospitals from 31 provinces as treatment and information centers for hemophilia. Up to April 6, 2012, there are 10,164 treatment cases of hemophilia in the information management system (12). Besides, in 2010, the Ministry of Health launched hemophilia diagnosis and replacement therapy training. The country's leading experts on hemophilia were invited to give lectures on the basics, diagnosis and treatment of hemophilia, hemophilia treatment-related blood transfusion and blood products by video conference, or other means, in order to improve the diagnosis and treatment level of hemophilia by the clinical doctors across the country. As a consequence, the training taught more than 8,500 doctors. Meanwhile, the domestic supply of coagulation factor VIII has markedly improved over the past two years with an annual total production of coagulation factor VIII of 39.7 million bottles (based on 200 units per bottle), meeting the basic clinical needs of patients with hemophilia (13). But still there is a big supply gap for coagulation factor VIII in rural regions of China. Furthermore, many hemophiliacs can not afford to pay high drug costs for a long treatment (14).

3. The medical insurance policies of rare diseases and orphan drugs in China

Haffner studied the changing nature of approved orphan products and disease indications by the Food and Drug Administration (FDA) of the USA from 1983 to 2003 (15), which showed there were approximately 85% orphan designations for the treatment of serious and/or life-threatening diseases, the highest percentage (31
% of orphan designations are for rare forms of cancer, while metabolic disorders represent the second largest group of orphan designations (11%); the majority of rare diseases are chronic, however a small number of products have been developed for single-occurrence diseases or emergency medicine for such conditions as acute smoke inhalation or lead poisoning.

The Chinese medical insurance system generally consists of the basic social medical insurance system, public medical insurance and commercial health insurance. Although the Chinese medical insurance system still does not establish special insurance programs for rare diseases and orphan drugs, it has covered some major diseases or specific diseases which include rare diseases.

3.1. The basic social medical insurance system

The basic social medical insurance system is jointly composed of Urban Employees' Basic Medical Insurance Scheme (UEBMIS, initiated in 1998), Urban Residents' Basic Medical Insurance Scheme (URBMIS, 2007), and New Rural Cooperative Medical Insurance Scheme (NRCMIS, 2003), respectively covering urban employees, urban non-employees, and the rural population (16). In 2008, the Fourth National Health Services Survey (NHSS, 2008) was conducted all over China. A total of 56,456 households or 177,501 people were investigated. Based on the NHSS result, as of June 2008, 87.1% of residents investigated were covered by government or collectively-run health insurance. In urban areas, coverage rates of basic medical insurance for urban employees and urban residents were 44.2% and 12.5%, respectively. The coverage rate of the NRCMIS for rural residents reached 93.0% (17). In 2010, the three schemes of basic social medical insurance covered over 1.2 billion people according to the China Human Resources and Social Security Yearbook (working volume).

We analyzed the national policies and implementing rules of 25 local governments for the three basic social medical insurance schemes. The common characteristics of the operations of the three schemes have three aspects. First, the three schemes require those eligible people to pay a premium; second, they design the deductible and the ceiling and reimbursement rates; last, they generally cover inpatient care and outpatient care. However, unlike the mandatory UEBMIS, URBMIS and NRCMIS have been running on a voluntary basis.

The three schemes function independently, differing in aspects related to financing, reimbursement, and expansion (16). In the UEBMIS and URBMIS, the social pooling fund mainly pays for inpatient costs in a ratio of reimbursement within a pre-defined band (above the deductible line but below the ceiling) and outpatient's expenditures incurred in the treatment of specific diseases or serious chronic or major diseases. The NRCMIS fund pays for inpatient costs in a ratio of reimbursement within a pre-defined band (above the deductible line but below the ceiling) and high outpatient expenditures. For the outpatient reimbursement of specific diseases or serious chronic diseases or major diseases, there are several ways for payment of the social pooling fund in the three schemes, which include the ceiling for a single major disease (without the deductible), the deductible line and the ceiling, reimbursement rate or in accordance with the local inpatient reimbursement policy.

Meanwhile, the central government gave a certain degree of autonomy to local governments in the implementation of the three schemes. The local governments also have the autonomy to determine the deductible, ceiling, and reimbursement ratio according to local economic and demographic status (16). As a result, the reimbursement level for eligible enrollees in every scheme varies greatly by region in China.

The UEBMIS requires enrollment of all urban employers and employees, who share in the responsibility of paying premium contributions. Premiums equivalent to 8% of employees' monthly payroll are contributed to medical insurance, with the employee contributing 2% and the employer providing the remaining 6%. All the premiums are divided into two parts, namely social pooling and individual accounts (16). In 2010, the average monthly payroll of urban employees at their posts is about 3,096 RMB. Namely the average annual premium per capita in the UEBMIS is about 248 RMB in China. For example, in 2011, the ceiling of the inpatient costs which are paid by the social pooling fund of Urban Employees in Beijing is 100,000 RMB per year, while it is 54,000 RMB per year in Urumqi. With regard to the premium in the URBMIS, every city has a different standard. In 2011, an unemployed adult urban resident who lives in Beijing needs to pay 600 RMB per year for the local pooling fund of the URBMIS in contrast to 372 RMB per year in Urumqi.

As for outpatient reimbursement for specific diseases in the NRCMIS fund, Guangzhou's policy for 15 specific diseases is to comply with inpatient reimbursement policy in 2012 (18), while Lanzhou's policy is that the ceiling of a single major disease is defined without the deductible. For instance, in Lanzhou city, the outpatient ceiling of hemophilia is 20,000 RMB per year, while the outpatient ceiling of arsenic poisoning or malaria is 1,000 RMB per year in 2012 (19). In Wuhan city (Hubei province), the ceiling of outpatient costs is 5,000 RMB per year in 2012.

Currently, in many Chinese cities, the UEBMIS and URBMIS define the kinds of specific diseases or serious chronic diseases or major diseases for outpatients, which mainly includes malignant tumors using chemotherapy or radiotherapy, severe uremic poisoning requiring hemodialysis, organ transplant requiring anti-rejection therapy, leukemia, hemophilia, aplastic anemia, systemic lupus erythematosus, Parkinson's disease, myasthenia gravis, ankylosing
spondylitis, etc. The specific diseases in the NRCMIS mainly include malignant tumors using chemotherapy or radiotherapy, severe uremic poisoning requiring hemodialysis, hemophilia, aplastic anemia, systemic lupus erythematosus, myasthenia gravis, thalassemia (Guangdong province), phenylketonuria and chronic C-viral hepatitis (Putian City, Fujian province), Keshan disease, Kashin-Beck disease, brucellosis, kala azar, cretinism, hydatid disease, skeletal fluorosis, arsenic poisoning, and malaria (Gansu province).

An interesting case study emerged in Tongling city (Anhui province) in 2011, located in central China. Tongling's government took some new measures for rare diseases in the UEBMIS and URBMIS. If an enrollee suffered from a rare disease, the reimbursement of his outpatient expenses was the same as the inpatient reimbursement. The orphan drug used will be reimbursed as a "Yi" tier drug in the UEBMIS. The medical expenses of the treatment of his rare disease which costs in other cities will be treated as local treatment expenses and get reimbursement (20).

What are our reimbursement policies for excess expenses above the ceiling? In order to further alleviate the persistent phenomenon of poverty resulting from catastrophic diseases, the central government and local governments encourage people to participate in the social insurance scheme for major or catastrophic diseases. For example, the urban enrollee also needs to pay a premium for the supplementary social pooling fund of major disease or the medical mutual pooling fund for huge medical inpatient costs. In 2009, Ministry of Health of China launched the children's medical insurance pilot plan for major diseases in rural areas. This pilot plan is a part of the NRCMIS. The major diseases of children have six types, including acute lymphoblastic leukemia, acute promyelocytic leukemia, congenital atrial septal defect, congenital ventricular septal defect, congenital patent ductus arteriosus, and congenital pulmonary valve stenosis. In 2011, this pilot plan covered over 7,200 children with leukemia who got 65% reimbursement and 22,600 children with congenital heart diseases who got 78% reimbursement (21).

In the early months of 2012, Ministry of Health of China began to build a major disease security pooling fund of the NRCMIS in a provincial or municipal pooling level. This fund will pay for the medical expenses of 12 major diseases, such as hemophilia, chronic myeloid leukemia, esophageal cancer, gastric cancer, colon cancer, rectal cancer, etc. Currently, hemophilia is covered by the UEBMIS and URBMIS from 18 provinces and is reimbursed by the NRCMIS from 16 provinces (21).

3.2. The public medical insurance program

The public medical insurance program (state-funded public medical program) is provided by the government to employees working in state agencies, such as civil services. This program is a kind of Free Medical Service program which is financed by the central and local governments according to the number of eligible enrollees. If the public medical insurance expenses in a state agency exceed the allocated funding, the excess medical expenses will be borne by the state agency. In the public medical insurance program, eligible enrollees in the state agencies do not have to pay a premium and a deductible. Meanwhile, they can get about 80% reimbursements without a ceiling (22). This program has low out-of-pocket costs and a high reimbursement rate.

The diseases covered in the public medical insurance program are decided by patient work agencies and the local administrative department. As a matter of fact, this insurance program covers some rare diseases, but public medical insurance only accounts for a small proportion in the Chinese medical insurance system. The coverage rate of the public medical insurance was 1.0% based on the data of NHSS in 2008. It is gradually being replaced by the basic medical insurance for urban employees (22).

3.3. Commercial health insurance

Commercial health insurance in China serves as a supplement to social medical insurance, targeting mainly the upper class. These plans have high premiums and reimbursement rates (16). According to the result of NHSS in 2008, the coverage rate of commercial health insurance was 6.9% in the investigated people. The per capita premium for commercial health insurance was 858 RMB, compared with 16 RMB for the NRCMIS and 186 RMB for the URBMIS (17). Commercial health insurance categories mainly cover particular critical illness, medical cost reimbursement insurance, and medical allowance insurance for accommodation fees related to hospitalization (16). As an illustration, there is a medical insurance plan for lifetime major diseases in Taikang Life Insurance Co., Ltd., Beijing, China. A male enrollee in the plan for lifetime major diseases may have a choice to pay a premium of 60,000 RMB once or 3,000 RMB/year up to 20 years. The ceiling of the medical cost of a lifetime major disease is 100,000 RMB.

Currently, there are more than 100 commercial insurance companies in China, offering around 200 kinds of insurance for patients with critical illness. In 2007, China Insurance Association issued "The Operating Regulation of the Insurance for Critical Illness". This regulation defined 25 types of major diseases such as Parkinson's disease, primary pulmonary hypertension, aplastic anemia, motor neuron disease, cancer, etc. Meanwhile, it also stated that the commercial insurance companies may exclude genetic diseases, congenital malformations, deformation or chromosomal abnormalities diseases and chronic lymphocytic leukemia, and Hodgkin's disease (23).
4. The rapid development of social supportive activities for rare diseases or orphan drugs in China

From 2003 to present, there has taken place a series of charity or academic activities focusing on rare diseases or orphan drugs in China. In 2003, Glivec® International Patient Assistance Program (GIPAP), a disease relief program, was launched by the China Charity Federation and Novartis Pharmaceuticals in China. Novartis Pharmaceuticals donated Glivec (imatinib mesylate tablets) to patients with chronic myelogenous leukemia (CML) or gastrointestinal stromal tumor in China. Currently, the price of Glivec (60 Capsules/box, 0.1g) is around 12,000 RMB. Generally, a CML patient needs to take 2 boxes of Glivec every month, namely 24,000 RMB/month, and 288,000 RMB/year. If a CML patient is supported by GIPAP, he or she may pay 72,000 RMB to buy 6 boxes of Glivec for the first 3 months, followed by free payment of Glivec for the next 9 months. For urban residents with low-incomes below the local poverty line or rural destitute family members, they may apply to get all free Glivec through this program. In July 2009, the China Charity Federation launched the charitable donation program of Cerezyme ® (imiglucerase for injection). Genzyme Corporation donated Cerezyme (valued at 200 million RMB) to help Chinese patients with Gaucher’s disease. Cerezyme received SFDA approval to market in November 2008 in China. The China Charity Federation built an assistance foundation for rare diseases in 2009 and started the β-thalassemia patient assistance project of deferasirox (Exjade®) in 2010. In 2011, Qingdao Municipal Charity Federation and Bayer jointly launched the "Little Sunflower Hemophilia Charity Fund". This fund has 1 million RMB to support children with hemophilia who need to use Kogenate® FS (recombinant coagulation factor VIII for injection) (24).

Meanwhile, some Chinese patient organizations for rare diseases were also set up one by one, e.g., the Home of Babies of the Moon – the China Albinism Association (http://www.albinism.org.cn), Beijing Rare Disease Care Center of the Hemophilia Home of China (http://www.xueyou.org), the Neuro-Muscular Disease Association of China, LAM-China (Lymphangio leiomyomatosis, http://www.lamchina.org), China TSC Together (tuberosclerosis complex, http://www.tscchina.org) and the China-Dolls Care and Support Association (osteoogenesis imperfecta, http://www.chinadolls.org.cn). The China-Dolls Care and Support Association was founded in 2007, which is a non-profit, non-governmental organization for people with osteogenesis imperfecta. The word "china" here has a dual meaning. One is porcelain, signifying that these patients are as fragile as porcelain. The other is the country China, emphasizing those with this disease are also Chinese citizens, and cannot be ignored and should not be discriminated against (1). Moreover, academic activities and organizations for rare diseases were launched. In April 2010, the China Charity Federation, China Health Education Center and Tsinghua University in Beijing sponsored "2010 Symposium on Rare Diseases". In October 2010, China's first rare disease control association was established in Shandong Academy of Medical Sciences, namely Shandong Rare Disease Prevention Association. In February 2011, a rare disease specialist Medical Association Shanghai Branch was established. In 2010 and 2011, China Central Television (CCTV) broadcasted a series of programs about the painful situation of patients with rare diseases and the shortage of orphan drugs in the "Economy 30 Minutes", "Focus" program. Public awareness of rare diseases and orphan drugs is increasing fast.

5. Obstacle factors of access to orphan drugs in China

"Orphan drugs" are medicinal products intended for diagnosis, prevention or treatment of rare diseases. These drugs are called "orphan" because the pharmaceutical industry has little interest or is reluctant to invest in developing and marketing products intended for only a small number of patients suffering from very rare conditions. For the drug companies, the cost of bringing a rare disease medicinal product to the market would not be recovered by the expected sales of the product (25). Although Chinese pay more attention to rare diseases and orphan drugs, there are three key obstacle factors which affect improving accessibility of orphan drugs for patients with rare diseases.

5.1. No definition of rare diseases and orphan drugs

Currently, there is not a clear definition of rare diseases and orphan drugs in China. In this case, it is very difficult for people including many health providers that can clearly identify a rare disease or a common disease and an orphan drug or a general drug. Certainly, efficiency and effectiveness of medical demand and supply information communication of rare diseases and orphan drugs between the different market players will be weakened. An appropriate example can be founded in many hospitals in China. The hospitals do not establish the orphan drug formulary or alternative formulary of orphan drugs and special management system for rare diseases and orphan drugs, so that the shortage of orphan drugs and high medical risk of rare diseases are very likely to occur (23). Fortunately, the Ministry of Health in China is aware of the treatment problems and begins developing a clinical treatment pathway for some rare diseases, such as myelodysplastic syndrome, chronic myeloid leukemia, chronic lymphocytic leukemia, diffuse large B cell lymphoma, hemophilia, autoimmune hemolytic anemia, children with acute lymphoblastic leukemia, children with acute promyelocytic leukemia, and musculararyotrophic lateral sclerosis (26).
5.2. Lack of effective incentive policies for orphan drugs

Compared with the orphan drug policies of the USA, EU and Japan, the Chinese incentive policies for rare diseases and orphan drugs which can be seen in the above chapter seem to lack a dynamic system. For push incentive policies, China is short of the direct cash investment in science research for rare diseases or orphan drugs, like special financial subsidies of the government for research grants, or tax credits for clinical research. For pull incentive policies, China does not set up useful market protection and assurance policies for orphan drugs, like market exclusivity, and a special market information network. Meanwhile, the supply and stockpile of orphan drugs have a low use frequency and high cost, so the drug wholesale and pharmacy also lack enough motivation to supply orphan drugs under no incentive policy. As a result, the output of orphan drugs is much less in China.

The Orphan Drug Act was passed in January 1983 in the USA, with lobbying from the National Organization for Rare Disorders and many other organizations. Under the law, companies that develop such an orphan drug (an orphan drug for a disorder affecting fewer than 200,000 people in the USA) may sell it with marketing exclusivity for 7 years, and may get tax credit for clinical research. For pull incentive policies, China does not set up useful market protection and assurance policies for orphan drugs, like market exclusivity, and a special market information network. Meanwhile, the supply and stockpile of orphan drugs have a low use frequency and high cost, so the drug wholesale and pharmacy also lack enough motivation to supply orphan drugs under no incentive policy. As a result, the output of orphan drugs is much less in China.

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Similar to the USA, EU, Australia, Singapore, Japan, South Korea, and Taiwan also set up legislation for rare diseases. For example, the Regulation on Orphan Medicinal Products was passed by the European Council in 1999. European Parliament and Council Regulation (EC) No 141/2000 came into force in April 2000. It also provided incentives for the development of orphan drugs (or other medical products for rare disorders) in the EU. Orphan drug status granted by the European Commission gives marketing exclusivity in the EU for 10 years after approval. The EU’s legislation is administered by the Committee on Orphan Medicinal Products of the European Medicines Agency (EMA). In May 2010, EMA had received more than 1,100 applications for orphan medicines. Out of these, 720 orphan designations have been granted, a success rate of 65%. A total of 62 orphan designated medicines have now been approved for use in the EU, giving treatment options for 53 different rare diseases (28).

5.3. Limited insurance coverage and reimbursement levels for rare diseases and orphan drugs

In the Chinese medical insurance system, the number of rare disease types covered is about 10-15, and the difference of disease coverage is obvious between different provinces. Currently, the central government of China claims the ceiling of inpatient expense for the social pooling fund in the three social insurance schemes is capped at six times of the local average payroll of urban employees or six times of the average disposable income of urban residents or eight times of the average net income of rural residents and is at least 60,000 RMB per year in 2012 (21). As an example of NRCMIS, it covered 0.836 billion people in 2011. The current issue is that the NRCMIS increases the enrollees’ access to medical service and stimulates their service utilization, but the enrollees’ medical expense burden of major disease was not alleviated significantly (29). As for the patients with hemophilia, they favor receiving outpatient treatment of hemophilia as opposed to inpatient treatment (14). Generally speaking, an average medical cost of 10-year-old children with hemophilia is about 60,000 RMB per year (30). The ceiling of outpatient expense for the social pooling fund in the three social insurance schemes is from 2,000 RMB per year (Shijiazhuang, Hebei province) to 45,000 RMB per year (Fushun, Guangdong province) in the different regions in China. As for CML, the medical expense burden of CML is still huge for the patient, although the medical costs can be paid partly by the social insurance schemes.

In a previous study, our group reported that the inpatients’ average medical cost of 11 rare diseases in the public hospital is 6,405.69 RMB and the average duration of stay in the hospital is 11.15 days, based on an analysis of the data released in China Health Statistics Yearbook from 2004 to 2008. In the corresponding period, the average per capita disposable income of an urban resident was 12,248.14 RMB and the average per capita net income of rural households was 3,735.86 RMB. For a disease, if the household’s financial contribution is capped at six times of the local average payroll of urban employees or six times of the average disposable income of urban residents or eight times of the average net income of rural residents and is at least 60,000 RMB per year in 2012 (21). As an example of NRCMIS, it covered 0.836 billion people in 2011. The current issue is that the NRCMIS increases the enrollees’ access to medical service and stimulates their service utilization, but the enrollees’ medical expense burden of major disease was not alleviated significantly (29). As for the patients with hemophilia, they favor receiving outpatient treatment of hemophilia as opposed to inpatient treatment (14). Generally speaking, an average medical cost of 10-year-old children with hemophilia is about 60,000 RMB per year (Shijiazhuang, Hebei province) to 45,000 RMB per year (Fushun, Guangdong province) in the different regions in China. As for CML, the medical expense burden of CML is still huge for the patient, although the medical costs can be paid partly by the social insurance schemes.

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As a matter of fact, the current Chinese medical insurance system is characterized by low premiums and large coverage, but its reimbursement for rare diseases and orphan drugs is limited.

6. Conclusion

Everyone should have equal rights of accessibility to basic health care opportunities. Under the condition
of a market economy, it is critical that the government should enact economic incentives to encourage drug companies to develop and market medicines for the many neglected and "orphaned" rare disease patients and ensure rare disease patients access to drugs and medical care.

All things considered, to raise the accessibility of orphan drugs in China, it is necessary to define basic concepts, list rare diseases and orphan drugs and build a systematic incentive mechanism for orphan drugs, design a special response system for orphan drugs in the medicine and health system supply, and establish a collaboration model of economic support between such parties as the government, enterprise, non-governmental organizations, and patients to reduce the patients' economic burden.

Acknowledgements

This work was supported by a grant from the National Natural Science Foundation of China (70903025) and a grant from the author's institution (HUST 2012QN005).

References


(Received February 1, 2012; Revised May 8, 2012; Accepted May 11, 2012)
The characterization and role of leukemia cell-derived dendritic cells in immunotherapy for leukemic diseases

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Summary

Usually, an effective anti-leukemia immune response cannot be initiated effectively in patients with leukemia. This is probably related to immunosuppression due to chemotherapy, down-regulation of major histocompatibility complex (MHC) II molecules, and the lack of co-stimulatory molecules on dendritic cells (DC). In light of this problem, some methods had been used to induce leukemia cells to differentiate into mature DCs, causing them to present leukemia-associated antigens and activating naïve T cells. Furthermore, leukemia-derived DCs could be modified with tumor antigens or tumor-associated antigens to provide a new approach to anti-leukemia therapy. Numerous studies have indicated factors related to the induction and functioning of leukemia-derived DCs and the activation of cytotoxic T-lymphocytes (CTLs). These include the amount of purified DCs, cytokine profiles appropriate for inducing leukemia-derived DCs, effective methods of activating CTLs, reasonable approaches to DC vaccines, and the standardization of their clinical use. Determining these factors could lead to more effective leukemia treatment and benefit both mankind and scientific development. What follows in a review of advances in and practices of inducing leukemia-derived DCs and the feasibility of their clinical use.

Keywords: Dendritic cell, leukemia cell, leukemia-derived DCs

1. Introduction

Dendritic cells (DCs), including myeloid DCs and lymphoid DCs, are derived from pluripotential hematopoietic stem cells. Although widely distributed throughout the body, only a few of DCs can be found in single organs and account for about 1% of the total amount of leukocytes in peripheral blood (1). The lack of DCs is partially related to development of some solid tumors or leukemias (1). Therefore, DCs have been studied to treat hematologic malignancies (2). Since patients with leukemia have small amounts of DCs in the blood and these DCs have poor clinical efficacy, researchers have focused on finding methods to increase the numbers of DCs to induce leukemia-specific CTLs. Several methods have been used to induce leukemia cells to differentiate into mature DCs, and these leukemia-derived DCs are capable of presenting leukemia-associated antigens and are used clinically (3).

This review summarizes current understanding of DC functioning in patients with leukemia, the anti-leukemia role of leukemia-derived DCs, and ways to induce DCs from leukemia cells.

2. Deficient immunity and a lack of DCs in patients with leukemia

Although intensive chemotherapy-based approaches, which include stem cell transplantation, have induced complete remission (CR) in 80% of patients with acute myeloid leukemia (AML), many patients still...
relapse due to the persistence of minimal residual disease (MRD), resulting in survival rates of 30-40%. Obviously, the leukemia-bearing host is immunologically tolerant to the remaining leukemia cells and therefore fails to eradicate the disease (4). The lack of co-stimulatory molecules on the surface of leukemia cells, as evident in the low expression of CD40 and CD80 and other molecules, can lead to the insufficient recognition and lack of presentation of specific antigens and failure to activate T cells (5-8). The expression of CD40 is considered to be crucial for T cell activation and expansion, so the absence of CD40 on blasts might be especially responsible for the insufficient recognition of blasts by the immune system in patients with AML (5).

Antigen-presenting cells (APCs) are devoid of co-stimulatory molecules and thus fail to activate T cells to start an effective anti-tumor immune response (9-11). At the same time, growing malignant tumor cells can secrete many immunosuppressive factors such as interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF) that can lead to dysfunction of DCs, resulting in tumor-associated antigens not being effectively presented to lymphocytes (12,13). Moreover, immature DCs in patients with leukemia might induce regulatory or suppressive T cells, impairing the quality of the anti-leukemia immune response as would occur with normal immature DCs (14-16). Recently, other proteins have been found to correlate with immunity or the prognosis for patients with leukemia. For example, CD56 expression in AML with t(8;21) is associated with a significantly shorter duration of CR and survival (17). B7-2 is one of the most crucial factors in the prognosis of adult acute leukemia and has an important role in tumor immunity (18), and poliovirus receptor-related (PRR) proteins could play a role in leukemia. Patients with a high level of PRR1 or PRR2 expression exhibit a more favorable prognosis (19). Thus far, however, the role of these factors in compromised immunity and the lack of DCs is still being studied. Presumably, the mechanism of immune reaction in patients with leukemia is as shown in Figure 1. Overall, the rapidly growing body of data offers new insights towards understanding AML biology and it provides evidence that DC subsets and dendritopoiesis in vivo are affected by leukemogenesis and may contribute to leukemia's evasion of the immune system.

![Figure 1. Mechanism of immune reaction in patients with leukemia.](image-url)

Deficient immune responses and MRD in patients with leukemia contribute to immunodepression due to chemotherapy, the down-regulation of MHC-II molecules, the lack of co-stimulatory molecules on DCs, FasL-induced apoptosis of T cells, and inhibition of the functioning of T or DC cells by TGF-β, IL-10, and VEGF. Some cytokine profiles and other agents can induce primary leukemia cells or leukemia cell lines to differentiate into immature or mature DCs. Such leukemia-derived DCs are responsible for presenting leukemia-associated antigens and convert the naïve T cells into CTLs, which can be enhanced by genes, tumor-associated antigen peptides, or cellular tumor antigens. The IFN-γ and chemokines secreted by leukemia-derived DCs can also modulate the functioning of NK and CTL or induce the migration of naïve T cells to lymphocyte organs. The activated CTL will kill leukemia cells and induce remission in patients with leukemia. Although DCs are used to treat patients with leukemia, DC-mediated leukemia immunotherapy is thus far confined to animal and in vitro experiments, and many problems remain to be solved.
3. *Ex vivo generation of DCs*

3.1. DCs produced from primary leukemia cells

At present, DC amplification mainly focuses on the effects of different cytokine combination profiles. The most conventional cytokines include granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4), and tumor necrosis factor-α (TNF-α). The GM-CSF + TNF-α + IL-4 profile can induce CD34+ hemopoietic stem cells and CD14 + monocytes in healthy persons to differentiate into DCs (20). T cell immunity against autologous leukemia cells mediated *in vitro* by DCs from patients with AML might prove useful for immunotherapy of AML even in patients with CR (21,22). Peripheral blood cells of patients with chronic myelomonocytic leukemia (CMML) can also be induced to acquire DC characteristics upon culturing with GM-CSF plus IL-4 plus TNF-α. CMML-derived DCs are potent stimulators of the allogeneic mixed lymphocyte reaction (MLR) and may serve as a cellular vaccine to induce anti-tumor immunity in patients with CMML (23). Some researchers have successfully induced chronic myelogenous leukemia (CML) cells to become CML-DCs using GM-CSF and IL-4, and the CML-DCs produced appear no different from DCs produced from primary leukemia cells (Table 1). Usually, cytokines including GM-CSF can induce AML leukemic cells to become immature DCs and further develop into mature DCs after induction by IFN-α, IL-1β, IL-6, or prostaglandin E2 (PGE2) (30,31). The ability of leukemia-derived DCs to induce proliferation of allogeneic T cells increases significantly and offers a useful path to active immunotherapy for patients with AML (32-34). Other factors also affect AML-DC formation and functioning. For example, only AML primary cells expressing CD14 and TNF-α-R1 can be successfully induced to differentiate into DCs (AML-DCs) by cytokines (35,36). The FLT3-ITD (FLT3 internal tandem duplication) can inhibit AML-DC formation and down-regulate VEGF expression and induce the production of functional AML-DCs (34). Ganoderma lucidum polysaccharides (GL-PS) can stimulate the maturation of monocyte-derived DCs in patients with monocytic leukemia (AML-M4 and M5) (37). DCs are harder to cultivate from AML samples than from CML samples, and DCs cannot be obtained from some patients with AML by conventional methods and with low levels of expression of several key molecules.

Furthermore, myeloid-leukemic cells in myelodysplastic syndromes (MDS) can be induced to differentiate into leukemia-derived DCs. In all, 31% to 52% of leukemic blasts can be converted to leukemia-derived DCs and 39% to 64% of these DCs are mature (38,39). Lymphocytic leukemia cells can also be induced to differentiate into leukemic DCs (AML-DCs) by cytokines (38,39). Ganoderma lucidum polysaccharides (GL-PS) can stimulate the maturation of monocyte-derived DCs in patients with monocytic leukemia (AML-M4 and M5) (37). DCs are harder to cultivate from AML samples than from CML samples, and DCs cannot be obtained from some patients with AML by conventional methods and with low levels of expression of several key molecules.

### Table 1. Induced differentiation of DCs from primary leukemia cells

<table>
<thead>
<tr>
<th>Leukemia</th>
<th>Methods of producing DCs</th>
<th>DC differentiation capacity</th>
<th>Antigen-presenting ability</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>GM-CSF, IL-4, TNF-α, FL, CD40L</td>
<td>+, ++</td>
<td>+</td>
<td>28-40</td>
</tr>
<tr>
<td>ALL</td>
<td>GM-CSF, IL-4, CML-DCs</td>
<td>+</td>
<td>+</td>
<td>22</td>
</tr>
<tr>
<td>MDS</td>
<td>GM-CSF, IL-4, TNF-α, FL, CD40L</td>
<td>+</td>
<td>+</td>
<td>41</td>
</tr>
<tr>
<td>CLL</td>
<td>GM-CSF, IL-4, TNF-α, FL, CD40L</td>
<td>+</td>
<td>+</td>
<td>28-44-46</td>
</tr>
<tr>
<td>CMML</td>
<td>GM-CSF, IL-4, TNF-α, FL, CD40L</td>
<td>+</td>
<td>+</td>
<td>42, 43</td>
</tr>
</tbody>
</table>

* GM-CSF + IL-4 + TNF-α + FL; 3 GM-CSF + IL-4 + TNF-α + CD40L; 4 GM-CSF + IL-4 + TNF-α.
* In some other studies, 4-1BB, LPS, or HDAC inhibitors were used to promote the maturation of leukemia-derived DCs.
signs regarding the role of effective adjuvants in the induction or functioning of leukemia-derived DCs. In the presence of GM-CSF, TNF-α, and/or IL-4, the leukemia-derived DCs that are usually obtained display features of immature DCs, so immature DCs must be induced to differentiate into mature DCs. With CD40L as a maturing agent, leukemic immature DCs can differentiate into cells that can fulfill the phenotypic criteria of mature DCs (40). Ionomycin calcium (CI) A23187 can induce human granulocytes to take on DC-like characteristics (41). Histone deacetylase inhibitors (HdI) could potentially improve the differentiation of leukemia-derived DCs induced from bone marrow samples of patients with ALL, as indicated by the up-regulation of CD86 (+) CD80 (−) cells (42). The role of the 4-1BB ligand (4-1BBL) in the T cell response induced by AML-DCs was examined and addition of 4-1BBL to cocultures of AML-DC and T cells was found to induce a preferential increase in the proliferation of CD8+ T cells. 4-1BBL was found to be an effective adjuvant to enhance the T cell response elicited by AML-DC (43).

3.2. DCs produced from leukemia cell lines

K562 cells, a CML cell line that includes multidrug-resistant leukemia K562/A02 cells, has been used in attempts to induce leukemia-derived DCs with different cytokine profiles, but the cells differentiated into K562-DCs only after exposure to GM-CSF + TNF-α + IL-4, which activated lymphocytes to produce CTLs (44, 45). IFN-γ was also detected in the supernatant, which enhanced the activity of CTLs and enhanced the ability of NK cells to kill target cells. There are contradictory findings regarding the DC differentiation capacity of THP-1 cells. Although THP-1 cells have been noted to acquire DC-like properties upon stimulation with cytokines, THP-1 cells have a relatively low DC differentiation capacity since less than 5% of THP-1 cells express the classic myeloid DC marker CD1a (46, 47). Moreover, THP-1 DCs fail to function like DCs, as indicated by the absence of allogeneic T-cell stimulatory capacity. Other reports, however, indicated that THP-1 leukemia cell lines can be induced to differentiate rapidly into mature DCs when cultured in serum-free medium containing GM-CSF, TNF-α, and ionomycin. Cell line-derived mature DCs are capable of stimulating allogeneic CD4+ and CD8+ T cells, ultimately defining them as potent APCs (48). Mature DCs were induced from human monocytic cell THP-1 by treatment with IL-4, GM-CSF, TNF-α, and ionomycin, and some cells were pretreated with PPAR-gamma agonists. Results indicated that induction of junctional adhesion molecule (JAM)-A occurred during differentiation of human THP-1 DCs and was independent of PPAR-gamma and the p38 MAPK pathway (49).

KG-1 is a cytokine-responsive, CD34+ myeloid cell line derived from a patient with erythroleukemia. Recently, KG-1 cells have also been described as acquiring DC-like properties upon stimulation with cytokines or PMA ± CI. Although KG-1 cells respond to stimulation with a number of factors known to induce differentiation and/or maturation of DCs in vitro, they usually do not differentiate in response to LPS, CpG oligodeoxynucleotide, or CD40L. Only treatment with PMA and ionomycin (with or without prior culturing in GM-CSF and IL-4) induced morphological and phenotypic changes consistent with DC-like maturation, and even these maximally differentiated KG-1 cells showed lower levels of surface marker expression and ability to stimulate an allogeneic MLR compared to monocyte-derived DCs in vitro. Although KG-1 cells differentiate into cells with DC-like functional characteristics in vitro, they lack the potential of mature DCs in terms of key aspects of specific antigen-presenting cells (46,50,51). Cyclophilin A (CypA) is a ubiquitously distributed intracellular protein that can enhance DC differentiation and maturation by up-regulating CD11b and CD11c expression and also augment DC antigen uptake and antigen presentation (52). The HL-60 acute promyelocytic leukemia cell line is a multipotent cell line capable of differentiating into granulocytes (53,54), monocyte-macrophage-like cells (35,56), or eosinophilic granulocytes (57). HL-60 cells have also been found to be insensitive to cytokine-induced DC differentiation (58). Upon CI treatment, HL-60 cells rapidly up-regulated CD86 and demonstrated de novo expression of CD83, CD80, and CD54. Expression of CD40 and CD1a only became apparent after 72-96 h. In addition, CI treatment also resulted in a marked increase in APC function, as determined by enhanced allogeneic T cell stimulation capacity. However, the fact that HL-60 cells failed to express MHC class II molecules and down-regulated MHC class I molecules upon CI treatment suggests that they may have limited antigen-specific T-cell stimulatory capacity (58,59). A large number of cells with a typical dendritic appearance were observed after culturing with CI A23187 for 72 hours, and the expression of CD80 and CD86 was continuously up-regulated. Allo-MLR revealed that DCs derived from HL-60 cells treated with A23187 or plus rhIFN-γ stimulated the proliferation of allogeneic human T cells (60). The Monomac-6 human acute monocytic leukemia cell line exhibits a well-differentiated monocyte phenotype with phagocytic activity and the expression of the mature monocyte marker CD14 (61), though they are unable to differentiate into DCs.

The U-937 histiocytic lymphoma cell line also exhibits monocytic characteristics, displaying a monoblast morphology (62). In contrast to Monomac-6 cells, U-937 cells are able to acquire mature, monocyte-like morphologic and phenotypic characteristics.
Table 2. Induced differentiation of DCs from leukemia cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>DC differentiation capacity</th>
<th>Cytokine profile</th>
<th>DC markers</th>
<th>Induced CTL activity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>+</td>
<td>GM-CSF + IL-4 + TNF-α</td>
<td>+</td>
<td>++</td>
<td>33, 61-65</td>
</tr>
<tr>
<td>THP-1</td>
<td>+/-</td>
<td>GM-CSF + IL-4 + TNF-α, or + CI</td>
<td>+</td>
<td>+/-</td>
<td>68-79</td>
</tr>
<tr>
<td>KG-1</td>
<td>+/-</td>
<td>GM-CSF + IL-4 + TNF-α, or PMA + CI</td>
<td>+</td>
<td>+/-</td>
<td>66-67, 71-74</td>
</tr>
<tr>
<td>HL-60</td>
<td>+/-</td>
<td>CI or CI + IFN-γ, Insensitive to cytokines</td>
<td>+</td>
<td>+</td>
<td>80-82</td>
</tr>
<tr>
<td>U-937</td>
<td>–</td>
<td>GM-CSF + IL-4 + TNF-α</td>
<td>–</td>
<td>–</td>
<td>84</td>
</tr>
<tr>
<td>Monomac-6</td>
<td>–</td>
<td>GM-CSF + IL-4 + TNF-α</td>
<td>–</td>
<td>–</td>
<td>67-68, 83</td>
</tr>
<tr>
<td>MUTZ-3</td>
<td>++</td>
<td>GM-CSF + IL-4 + TNF-α</td>
<td>+</td>
<td>++</td>
<td>66,68, 85-93</td>
</tr>
<tr>
<td>NB4</td>
<td>+</td>
<td>ATRA; ATRA + GM-CSF, CD40L; PA + TNF-α</td>
<td>+</td>
<td>+</td>
<td>94, 95</td>
</tr>
</tbody>
</table>

upon stimulation with PMA. Despite its monocye-like characteristics, the U-937 cell line is unable to differentiate into DCs. The MUTZ-3 cell line is derived from the peripheral blood of a patient with AML (63) and was used to generate immature dendritic-like cells (MUTZ-3 DC) (64,65). Compared to monocyte-derived DCs, the MUTZ-3 cell line has been shown to downregulate CD14 in response to GM-CSF and IL-4 and to exhibit characteristics of CD34-derived DC precursors (46). The MUTZ-3 precursors acquire a more myeloid DC precursor phenotype with up-regulated expression of the differentiation markers CD11c, CD11b, CD11c, CD13, and CD45RO. Moreover, cytokine receptors that are associated with DC differentiation such as GM-CSF-R and TNF-α-RI and -RII are up-regulated. In contrast to other AML-derived cell lines, MUTZ-3 cells have a marked DC differentiation capacity since CD1a expression after DC differentiation ranges between 60% and 90% for MUTZ-3 IDC and LC, respectively (66,67). Moreover, MUTZ-3-generated IDC and LC also express intermediate to high levels of co-stimulatory, adhesion, and MHC class I and II molecules, indicating that MUTZ-3 IDC and LC exhibit a true DC phenotype (68). Importantly, MUTZ-3-derived IDC and LC may also mature further in the presence of cytokines or CD40L, resulting in up-regulation of costimulatory and adhesion molecules CD80, CD86, CD40, CD54, and HLA-DR and de novo expression of CD83 (69). All-trans retinoic acid (ATRA) can also induce the NB4 retinoic acid (RA)-sensitive promyelocytic leukemic cell line to differentiate into DC-like cells and these differentiated cells can activate T cells. Results suggest that the differentiation of NB4 cells by ATRA causes the cells to express DC markers and that ATRA-differentiated NB4 cells are able to present antigens to T cells (70).

Jin et al. investigated whether phosphatidic acid (PA) can induce NB4 cells to differentiate into DC-like cells and they found dioctanoyl-PA alone upregulated the expression of DC markers. The expression of DC markers on NB4 cells was facilitated by the overexpression of phospholipase D and upregulation was blocked by the addition of n-butanol, an inhibitor of PA production. The expression of CD11c, CD83, and CCR7 in PA-treated NB4 cells was further increased by TNF-α treatment. These results suggest that PA induces differentiation of NB4 cells into DC-like cells and that the upregulation of antigen-presenting cell markers is mediated by the activation of ERK and the downregulation of PML-RAR alpha levels (71).

To summarize, attempts at inducing K562, THP-1, KG-1, Monomac-6, U-937, MUTZ-3, and NB4 cells to differentiate into DC-like cells have been tried with a similar cytokine profile or special drugs. Although they were all induced to differentiate into DC-like cells with morphologic and phenotypic characteristics of DCs, K562, THP-1, KG-1, MUTZ-3, and NB4 cells have obvious potential as mature DCs in terms of key aspects of specific antigen-presenting capacity (Table 2).

4. Modified DCs for clinical use

4.1. Genetically modified DCs

Specific mRNA fragments encoding the tumor antigens can be transduced into DCs to generate DC vaccines. Methodologically, several recombinant DNA delivery techniques have been used. In one study, nucleofection and adenoviral transduction were compared in terms of their efficiency at transducing human MoDCs in vitro. The use of a fiber-modified adenovector may therefore be preferable to non-viral gene delivery systems for DCs that will be used in cancer immunotherapy (72). For example, Muller et al. successfully transduced all of the mRNA isolated from type-B leukemic cells into DCs, further activating CTLs to kill leukemic cells (73). Nikitina et al. utilized the adenovirus vector Ad2p53 to transduce the wild type p53 gene into DCs, further stimulating T cells to generate p53-specific CTLs capable of killing the corresponding K562 leukemic cell strain (74). Therefore, electroporation of mRNA-encoding tumor antigens is a powerful technique to charge human DCs with tumor antigens and could provide tumor vaccines (75). The genes encoding some cytokines, co-stimulators, and chemokines can also be transduced into DCs to generate DC vaccines, enhancing the immunogenicity of the cells and starting a specific anti-tumor immune response. The expression of CD40 in vitro is considered to be crucial for T cell activation and expansion (5). Moreover, its expression on blasts was
almost absent. Thus, the absence of CD40 or CD80 on blasts in particular might be responsible for the insufficient recognition of blasts by the immune system of patients with AML. Thus, enhancing the expression of CD80 on leukemia blasts could increase their costimulatory activity on autologous T cells (76).

4.2. DC vaccines modified with tumor antigens

Previous studies (77-79) found that there was extensive expression of leukemia-associated antigens (LAAs) such as the preferentially expressed antigen of melanoma, the receptor for hyaluronic acid-mediated motility, and Wilms' tumor gene (WT-1) on AML blasts in contrast to PBMCs from healthy volunteers. Greater expression of LAAs by AML-DCs was observed in comparison to AML blasts. The amount of tumor antigens expressed in leukemia-derived DCs will affect their ability to initiate an immune response (8).

Recently, other leukemia-associated antigens that are recognized by CTL in the context of HLA class I molecules have been identified. These include fusion gene products such as BCR-ABL and ETV6-AML1, proteinase 3, human telomerase reverse transcriptase, and cyclophilin B. These findings have led to various clinical trials involving peptide modification and DC therapy (80). Tumor lysates can be utilized to load DCs to generate DC vaccines. DCs with strong phagocytosis can take up and present effective constituents such as tumor-associated antigens (TAA) in lysates, the MHC-II molecules in oncocyte extracts and the minor histocompatibility antigens to activate CTLs and induce an immune reaction. Apoptotic tumor cells can also be utilized in DC loading. DCs can take up apoptotic bodies and further process and present tumor antigens to induce tumor-specific CTL action (81). Klammer et al. (82) used leukemic cells and DCs collected from the peripheral blood to produce fusion vaccines, and their results indicated that the fusion cells not only expressed tumor antigens but also expressed the surface markers and co-stimulatory factors of DCs. Fusion vaccines made from tumor cells and allogenic DCs mainly secrete IFN-γ, which can induce Th1 differentiation, while fusion vaccines made from syngeneic DCs can induce the differentiation of both Th1 and Th2. Since the anti-tumor immune response is mainly a Th1-induced cellular immune response, allogenic DCs are more suitable for fusion vaccine production. DC modification with cellular tumor antigens is relatively simple and easy, but it still has some disadvantages. For example, a large amount of tumor cells is required and there are large amounts and various types of non-associated antigens that can induce auto-immune diseases; the utilized antigen peptides cannot always induce optimal anti-tumor immune responses because of antigenic modulation, antigenic deletion, low antigen immunogenicity, and the difficulty of determining the stimulating dose. AML-DC vaccines might be provided by the addition of adjuvants such as LPS or CpG-rich oligodeoxyribonucleotide binding to TLR and induction of a greater Type 1 T cell response. AML-DCs strongly expressed TLR-2 and TLR-4, while TLR-9 was expressed at a lower level. In accordance with the TLR expression levels, DCs produced from patients with AML responded to the known microbial ligands peptidoglycan (PGN) and lipoteichoic acid for TLR-2 and LPS as a ligand for TLR-4 by producing TNF-α and IL-6. A response to the ODNs 2006 and 2216 binding to TLR-9 was only detected in AML-DCs (83).

5. Quantity and quality control of leukemia-derived DCs

The leukemic derivation of AML-DC may be indicated by the persistence of clonal cytogenetic aberrations in DCs or by coexpression of leukemic antigens on DCs. For example, proof of the clonal derivation of DC was obtained in five AML and four MDS cases (38) with a combined FISH/immunophenotype analysis (FISH-IPA). The clonal numerical chromosomal aberrations of the diseases were regularly detected along with DC markers, but not with all clonal cells being converted to leukemia-derived DCs (on average, 53% of blasts in AML or MDS). Instead, not all DCs had clonal aberration (on average, 51% of DCs). On average, only 57% of blasts in AML and 64% of blasts in MDS were converted to leukemia-derived DCs (DC (leu)).

In order to establish the value of DC vaccination in patients with leukemia, some consensus on quality criteria and immune monitoring is essential. Although leukemic DCs meet most quality criteria, the optimal level of maturation needed to elicit an immune response should be determined. The quality of DCs can be defined by morphological, immunophenotypic, and functional criteria (84).

Cut-off proportions of mature DCs, DC (leu), proliferating, CD4 (+), CD8 (+), and non-naïve T cells after DC-stimulation were predictive for the anti-leukemic-activity of stimulated T cells as well as a response to immunotherapy. Interestingly, ratios greater than 1 of CD4:CD8 or CD45RO:CD45RA T cells were predictive for anti-leukemic function after DC stimulation. In an attempt to further characterize the DC/DC leu-induced T cell response pattern, immunoscope spectratyping was used to detect T cell receptor (TCR) rearrangements in combination with functional flow cytometry and a non-radioactive fluorolysis assay. Results indicated that a combined strategy using spectratyping with functional tests might not only provide useful information about the specificity and efficacy of the induced T cell response but also pave the way to effective T cell clones for therapy (85).
The composition and quality of DCs after a mixed anatomic region, potentially leading to increased T cell delivery of a known amount of DCs to the desired administration circumvents this problem and allows T cell response has yet to be identified. Intranodal optimal route of administration providing a better carries the risk of damaging lymphatic tissues. An injection also requires specialized techniques and quantity of DCs to the required location, potentially this problem and it facilitates the delivery of a known lymph nodes. Intra-lymph nodal injection can avoid will affect the ability of the DCs to migrate to the injection for T cell induction, but injection dependence subcutaneous injection is preferable to intravenous regarding administration routes, intracutaneous or subcutaneous injection is preferable to intravenous injection for T cell induction, but injection dependence will affect the ability of the DCs to migrate to the lymph nodes. Intra-lymph nodal injection can avoid this problem and it facilitates the delivery of a known quantity of DCs to the required location, potentially enhancing the immune function of T cells. Nodal injection also requires specialized techniques and carries the risk of damaging lymphatic tissues. An optimal route of administration providing a better T cell response has yet to be identified. Intranodal administration circumvents this problem and allows delivery of a known amount of DCs to the desired anatomic region, potentially leading to increased T cell immunity.

Furthermore, the route of administration may determine the location of the primary immune response, the distribution of memory cells, and the ability to control the tumors at different sites in the body.

DCs were cultivated from the peripheral leukemic cells of patients with lymphoma who had undergone chemotherapy mobilization and then retransfused into the body after they were loaded with autologous lymphoma antigens. This approach was clinically efficacious and facilitated anti-tumor immunotherapy with DCs. In a study of 19 patients with AML, Choudhary et al. successfully cultivated DCs in vitro from the leukemic cells of 18 patients, and after incubation with autologous lymphocytes these DCs induced obvious cytolysis of autologous AML cells.

Roddie et al. reported the results of their Phase I/II clinical studies to treat acute leukemia by inoculating patients with autologous AML-DCs. They stimulated the leukemic cells of 5 patients with AML with GM-CSF + IL-4 for 4 days, GM-CSF + TNF-α for 3 days, and finally IFN-γ + poly (I : C) for 24 hours, and then they inoculated the 5 patients in the remission stage after chemotherapy with these cells. Their results showed that two patients still had CR 12 months after inoculation.

To summarize, the aforementioned groups were not inoculated with the same amounts of DCs; most were between 106 to 107. Inoculation timing was nearly the same and most patients were in remission after chemotherapy. Roddie et al. reported the results of their Phase I/II clinical studies to treat acute leukemia by inoculating patients with autologous AML-DCs. They stimulated the leukemic cells of 5 patients with AML with GM-CSF + IL-4 for 4 days, GM-CSF + TNF-α for 3 days, and finally IFN-γ + poly (I : C) for 24 hours, and then they inoculated the 5 patients in the remission stage after chemotherapy with these cells. Their results showed that two patients still had CR 12 months after inoculation (32). Li et al. inoculated 5 elderly patients with AML with AML-DCs as a second or third-line treatment, and 3 patients lived stably for 5.5 months to 13 months while 2 patients died from rapid progression and deterioration (98). Five end-stage patients with AML were subcutaneously injected with AML-DCs up to four times at a biweekly interval, and sufficient amounts of MNCs were collected in leukopenic patients. Large-scale production of AML-DCs in cell factories under GMP conditions yielded an adequate quantity of viable and mature AML-DCs.

In another clinical experiment, two groups of patients with CML in the chronic phase and the acute phase were inoculated 4 times with 3 × 106 and 15 × 106 CML-DC cells, respectively. Results revealed no obvious clinical therapeutic response. This was probably because all of the selected patients were in the advanced stage and the amount of the inoculated DCs was rather small (92). Thus, at least 10 × 106 DCs are required to elicit an immune response in patients with CML, and an autologous CML-specific T cell response has been detected. Additionally, infused CML-DCs induced the appearance of T cell clones expressing the same T cell receptor, suggesting that the immune repertoire included tumor-reactive T cells. Other groups treated patients with chronic-phase CML (CP-CML) with various leukemic antigen peptides, resulting in an apparent immune response and clinical response. Imatinib mesylate is currently used as the first line therapy for CP-CML. Although it selectively targets the ABL portion of BCR-ABL protein as a reversible tyrosine kinase inhibitor, it cannot kill the leukemic stem cells of CML. Immunity could be enhanced in patients with CML treated with imatinib by combining it with immunotherapy, so the immune response of innate and adaptive immunity in CML has been summarized. Development of such immunotherapeutic strategies would be a promising approach to treat patients with CML-CP treated with imatinib (96).
vaccinations has also been tested in animal models. Pawlowska et al. reported that tumor-lysate pulsed DCs effectively prevented mice from developing leukemia, but mice with established disease could not be cured (101).

Leukemic-specific T cell responses were detected in some patients with AML with administration of AML lysate-pulsed monocyte-derived DCs (102,103). Immunotherapy is thought to be most effective at eradicating MRD, which is not done by immune cells themselves because they have been compromised by high-dose chemotherapy or radiation therapy (104,105).

During early remission, immune responses seem to be largely MHC-restricted whereas later on the immune response shifts towards being non-MHC restricted (106). Patients with high levels of MRD after chemotherapy are particularly likely to benefit from an early start of a vaccination or with the adoptive transfer of leukemia-specific T cells enhanced ex vivo (89,107,108). Important evidence suggesting that only mature DCs are suitable for use is because loading immature DCs with antigens will lead to the functional quiescence of T cells resulting from T cell removal or amplification of only regulated T cells (15). An important argument for the use of only mature DC is that antigen-loaded immature DCs silence T cells either by eliminating them or by enhancing regulatory T cells (109) or prolonging periods of maturation, hampering their capacity to stimulate a Th1 response (110). These results demonstrate that AML-DCs have certain therapeutic effects in patients whether they are in remission or not. All of these findings confirm that retransfusion of autologous DC vaccines is safe and practical and can stimulate an immune response in patients and enhance their immunity. Claxton et al. co-cultured CML-DCs with autologous T cells at a rate of 1:10 for 3 days and then retransfused all of these cells back into patients with CML; this resulted in control of leukocytosis in one patient for several months afterwards. The second patient took hydroxyurea (HU) orally before DC retransfusion and the Ph+ cells showed no obvious decrease after 3 DC transfusions (111).

Although DC therapy has been successful in inducing specific anti-tumor immune responses, data on clinical responses are not yet conclusive and most studies only report minimal antitumor effects (89,112). New approaches are needed to warrant use of DC vaccines in treating leukemia (113), and more strategies are required to sensitize residual leukemic cells. This aspect warrants further investigation in order to increase the immune stimulatory effect of leukemic DCs (114). Areas that are ripe for study are the components needed to produce DCs for therapy, including their culture and cytokine profile, antigen loading and delivery, and the potential for combination of DC-based immunomodulatory strategies (115). New methods of LAA antigen loading and maturation of leukemia-derived DCs should be explored their efficacy. Evolving DC-based therapeutic strategies addressing multiple components of tumor-immune system interactions may yield substantial benefits for patients (116,117).

7. Conclusion and perspectives

As specific APCs with important immunomodulatory action, DCs are a new concept of anti-tumor vaccine with promising prospects. Specific CTLs induced by DCs can initiate specific immunity and provide a new approach to anti-tumor immunotherapy. That said, the exact mechanisms for differentiation of DCs and the molecular mechanisms of DC functions are still unclear, so ways to regulate DCs to function selectively and thoroughly must be investigated further. Furthermore, studies on DC-mediated leukemia immunotherapies are still confined to animal and in vitro experiments, and there are many problems remaining. These include ways to obtain a large amount of purified DCs, the appropriate cytokine profiles for inducing DCs from leukemic cells, efficient methods of activating CTLs, reasonable approaches to DC vaccines, and the standardization of their clinical use. Moreover, whether CTLs induced by DCs cause adverse effects must be answered and ways to evaluate the characteristics of mature or immature DCs must be ascertained. Although studies of leukemic DCs provide new insights towards understanding both leukemogenesis and the physiology of DCs and studies on DCs used in leukemia treatment have made considerable progress, there is a fine line between immune tolerance and activation. The availability of leukemia-derived DCs and their capacity to enhance tumor recognition is a promising approach to immunotherapy for AML and other kinds of leukemias. The design of a clinical DC-based vaccine immunotherapy protocol requires a concise functional characterization of DCs as well as reflection on the crucial role of routes and timing of vaccine delivery to ensure delivery of specific cytotoxic effectors and helper T lymphocytes. If DC-based therapy is to benefit patients, it will probably do so for patients with minimal residual disease following or accompany other established therapies. The optimization of DC-based vaccines is on par with the development of sensitive techniques to monitor minimal residual disease and reliable methods of measuring patient responses to DC vaccines. Effective provision of leukemia-derived DCs in anti-tumor biotherapies will benefit both mankind and scientific development.

Acknowledgements

This work was supported by the Key Projects of the Shandong Science Foundation (2007GG2002023), the Shandong 1020 Project (2008-2), the Natural Science Foundation of Shandong (Y2008C165), and the Natural Science Foundation of China (30810444).
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(Received December 26, 2011; Revised March 28, 2012; Accepted April 13, 2012)
Primary biliary cirrhosis and liver transplantation

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1. Introduction

Primary biliary cirrhosis (PBC) is an immune-mediated chronic progressive inflammatory liver disease, predominantly affecting middle-aged women, characterized by the presence of antimitochondrial antibodies (AMAs), which can lead to liver failure. Genetic contributions, environmental factors including chemical and infectious xenobiotics, autoimmunity and loss of tolerance have been aggressively investigated in the pathogenesis of PBC, however, the actual impact of these factors is still controversial. Survival of PBC patients has been largely improved with the widespread use of ursodeoxycholic acid (UDCA), however, one third of patients still do not respond to the treatment and proceed to liver cirrhosis, requiring liver transplantation as a last resort for cure. The outcome of liver transplantation is excellent with 5- and 10-year survival rates around 80% and 70%, respectively, while along with long survival, the recurrence of the disease has become an important outcome after liver transplantation. Prevalence rates of recurrent PBC rage widely between 1% and 35%, and seem to increase with longer follow-up. Center-specific issues, especially the use of protocol biopsy, affect the variety of incidence, yet, recurrence itself does not affect patient and graft survival at present, and retransplantation due to recurrent disease is extremely rare. With a longer follow-up, recurrent disease could have an impact on patient and graft survival.

Keywords: Primary biliary cirrhosis, liver transplantation, ursodeoxycholic acid

Although numerous studies have revealed that environmental factors, inherited genetic predispositions and loss of tolerance appear to contribute to its autoimmune pathogenesis, the actual impact of these factors is still controversial (7).

Median survival in untreated individuals had been reported to be 7.5 to 16 years (1,8), however, it has largely improved since the introduction of ursodeoxycholic acid (UDCA) therapy and liver transplantation (9-12). Early recognition of the disease with serological tests and treatment with UDCA at an early stage of the disease has not only enabled patients to enjoy equivalent life expectancy to that of the normal population, but also dramatically decreased the need for orthotopic liver transplantation (13,14). However, the beneficial mechanisms of UDCA treatment are still incompletely understood, since about one third of patients fail to adequately respond to UDCA monotherapy, and liver transplantation is still a last resort for those with end stage PBC (2). While the outcome of liver transplantation for PBC is excellent,
recurrent disease after liver transplantation for PBC occurs in a considerable number of recipients and is currently a matter of concern (15-17).

The aim of this review is to summarize recent studies of PBC with special reference to liver transplantation.

2. Natural history and characteristics of PBC

2.1. Epidemiology

In a systematic review of studies of PBC frequency, the incidence and prevalence of PBC was reported to range from 0.7 to 49 per million and 6.7 to 402 per million, respectively (18). The highest incidence and prevalence rates were reported from the UK (19,20), Scandinavia (21), Canada (22), and the US (23), all in the northern hemisphere, while the lowest were found in Australia (24). At the same time, the incidence was reported to be increasing with time (25).

Most autoimmune diseases are predominant in female patients, and in PBC, this predominance is especially striking, the ratio of affected females to males is as high as 10:1 (26). The equivalent detection of AMAs between females and males in the general population might suggest either that the diagnosis of PBC might be suspected more frequently in women than in men or that progression from loss of tolerance to clinical liver disease might be more common in female patients (26).

2.2. Pathogenesis

Precise pathogenic mechanisms of developing PBC remain unknown. However, the development of PBC is believed to result from a combination of multiple genetic factors interacting with environmental triggers (27). Three important observations must be taken into account to understand the current pathogenic considerations of PBC. First, appearance of AMAs before developing liver disease suggests that loss of tolerance to the mitochondrial autoantigen is an initial event and could be independent of the development of liver disease. Second, although the autoantigen is present ubiquitously in all nucleated cells, the immune response is restricted to epithelial cells of intrahepatic bile ducts and less frequently to cells of salivary and lacrimal glands. Finally, recurrence of PBC after liver transplantation supports the idea that bile duct epithelial cells are a genetic target and are not unique to the naïve liver with PBC (4).

Genetic contributions to PBC Genetic factors have an impact on PBC pathogenesis that is stronger than in nearly any other autoimmune disease (28,29). Observations that 1-6.4% of patients with PBC have at least one family member manifesting the disease (30-32), and that a concordance rate of 63% was seen in monozygotic twins (as opposed to null concordance in dizygotic twins) (33,34) shows the substantial genetic effect on disease susceptibility.

Allelic variations in the human leukocyte antigen (HLA) genes, located in the highly polymorphic major histocompatibility complex (MHC), have been revealed to have an association with a large majority of autoimmune diseases (35). In PBC, the most commonly detected HLA association has been with the MHC class II DRB1*08 allele family (36), especially DRB1*0801 in European and North American Caucasians (37-39) and DRB1*0803 in the Japanese (40,41). A protective association has been demonstrated with DRB1*11 and DRB1*13, but once again, significant population differences were observed (36,38).

Association has also been reported with polymorphisms of genes involved in innate or adaptive immunity. Allelic variations of tumor necrosis factor alfa (TNF alfa) (42,43), cytokotic T lymphocyte antigen 4 (CTLA4) (42,44), and inteleukin 12 (IL12) (45) have so far been reported to be associated with PBC development in previous studies.

Environmental factors Despite the above mentioned strong evidence for genetic contributions, epidemiological studies have since early times suggested a role of environmental factors in triggering or deteriorating PBC (46). A significant effect of environmental factors was supported by the identification of geographic disease "hot spots" (industrial, coal mining, and polluted areas) in previous studies (27,47-49). Additionally, hormone replace therapy (50), frequent use of nail polish (50), and smoking (50-52) have been associated with developing and accelerating PBC. These findings led many to hypothesize the possible association of exposure to chemical environmental compounds and xenobiotics (including drugs, pesticides, or other organic molecules) with PBC if those chemicals are excreted into bile and thereby concentrated in the biliary tree (53). Xenobiotics may affect the pathogenesis of PBC by triggering autoimmune reactions with a potential direct toxic effect on cell/protein or a potential modification of host proteins to form neoantigens (54,55). Infection is another important environmental factor (56). A significantly higher prevalence of recurrent urinary-tract infections than usual in patients with PBC has been reported (50,57). Additionally, a sequence similarity between the E2 enzyme of the pyruvate dehydrogenase complex (PDC-E2), the main autoreactive antigen recognized by AMA identified in PBC, and bacterial proteins has been found in experimental studies (58).

Among various pathogens investigated including Escherichia coli (59), Helicobacter spp (60), Mycoplasma (61), Chlamydia (62), and human beta retrovirus (63), Novosphingobium aromaticivorans had the highest homology to PDC-E2 (64,65). Despite these intriguing associations, no compelling data have
been provided to show that one individual infectious agent can reproducibly be detected in patients with PBC. Thus the model of infections as a cause of PBC is supported by little direct evidence (27).

Autoimmunity and loss of self-tolerance The significant predominance of female patients, frequent co-existence of other autoimmune diseases both within individuals and among families, and most importantly, the presence of autoantibodies to self-mitochondrial proteins have led PBC to be referred to as a "model autoimmune disease" (27,66). The predominant autoreactive antibodies in PBC are AMAs, which, with a high sensitivity and specificity, are actually diagnostic for PBC when detected in serum (67). Follow-up data from AMA-positive individuals without signs of liver disease suggest that autoantibodies appear several years before onset of PBC and have a high predictive value for developing the disease (68).

The identified targets of AMA are all members of the family of 2-oxo-acid dehydrogenase complexes (2-OADC). This includes PDC-E2, the branched chain 2-oxo-acid dehydrogenase complex (BCOADc-E2), the 2-oxo-glutaric acid dehydrogenase complex (OGDC-E2), and the dihydrolipoamide dehydrogenase binding protein (E3BP), which all localize within the inner mitochondrial matrix, catalyzing oxidative decarboxylation of keto acid substrates (69,70). The targeted E2 subunits all have a common N-terminal domain containing single or multiple attachment sites for a lipoic acid cofactor to lysine. Previous studies have demonstrated that the dominant epitopes recognized by AMA are all localized within these lypoyl domains of the target antigens (71,72).

In addition to AMAs, autoreactive CD8 and CD4 T cells to the same PDC-E2 domain have been identified both in peripheral blood and within the liver of PBC patients (73), and the same accounts for the dominant autoreactive B cells (74). CD8 T cells isolated from livers of PBC patients have been found to exert cytotoxicity against PDC-E2 pulsed autologous cells, supporting the hypothesis of a T cell response contributing to bile duct injury in PBC (75).

It remains a mystery how PDC-E2 and other epitopes localized in the inner membrane of mitochondria become targets for autoimmune injury in PBC. Substantially lower rates of CD4 CD25 high regulatory T cells, acting to prevent autoreactivity, could be an important factor in the breakdown of tolerance (76,77). Increased amounts of polyclonal IgM and hyper-responsiveness to the cytosine-phosphate-guanine dinucleotide motif (78), and enhanced natural killer cell (73,79) and monocyte responses (80), all which are found in PBC patients, also support the role of innate immunity in developing PBC. Another hypothesis is that modifications of 2-OADC by xenobiotics may alter these self-proteins to cause a breakdown of tolerance facilitating an autoimmune response (81).

Once tolerance to AMAs is lost, additional mechanisms entailed in the immune response to a ubiquitous autoantigen begin to be unraveled. These lead to specific injury of biliary epithelial cells and seem to be linked to unique processes of apoptosis (54,82). Unlike other cell types, PDC-E2 seems to remain intact in bile-duct cells after apoptosis, thus preserving its immunogenicity (83). It was found to exist within apoptotic blebs and to be accessible to AMAs and local antigen-presenting cells (84). Additionally, in-vitro experiments revealed an intense and specific immune response when macrophages of PBC patients were combined with apoptotic blebs on biliary epithelial cells and AMAs (85). However, recurrence of PBC after liver transplantation suggests that this scenario is not an intrinsic defect of bile-duct cells of affected individuals but is a feature of biliary epithelia in general, not seen in other epithelial cells.

2.3. Diagnosis

Increased awareness of the disease and the increasing availability of diagnostic tools, in particular serological testing, have led to a more frequent and earlier diagnosis of PBC (25). Most recently, more than half of the patients diagnosed with PBC are asymptomatic at first presentation (86,87). The diagnosis of PBC can be established in case two of the following three criteria are fulfilled: a concentration in serum of AMAs at titers of 1:40 or higher; an unexplained rise in the amount of alkaline phosphatase of at least 1.5 times the upper limit of normal for more than 24 weeks; and compatible liver histological findings, especially non-suppurative cholangitis and interlobular bile duct injury (88-90).

Serum alkaline phosphatase (ALP), gammaglutamyl transpeptidase (GGT), and cholesterol are commonly elevated in patients with PBC, however, the presence of AMA could be the first expression of PBC and could be found in an asymptomatic stage or in the absence of abnormal biochemical tests (89,90). To elucidate the frequency and antigen specificity of AMAs in the asymptomatic population and to identify patients with early PBC, Mattalia et al. (91) investigated the prevalence of AMA in a healthy population, and found that approximately 0.5% of the population was positive for AMA. Importantly, the pattern of reactivity to PDC-E2 in non-PBC individuals differed from that found in PBC patients in most of the AMA positive sera, demonstrating that AMA as a "natural" autoantibody is different from a "pathological" autoantibody in PBC patients. Mitchison et al. (92) evaluated and followed biochemical/histologic/clinical outcomes of AMA-positive individuals with normal liver function, which revealed 24 out of 29 patients developed biochemical evidence of cholestasis and 22 became symptomatic, confirming a high predictive value of positive AMA testing for the development of PBC during a 10-year
follow-up (68). By contrast, individuals with AMA-negative PBC diagnosed on the basis of elevated ALP and liver histologic findings comprise 5% of the PBC population and manifest a similar course compared to their seropositive counterparts (93).

The need to undergo liver biopsy in PBC is controversial, although most clinicians agree that this procedure is valuable for disease staging, particularly in clinical trials. From a diagnostic point of view, liver biopsy specimens are not required when the other two criteria are fulfilled (89,90).

2.4. Clinical features and natural history of PBC

The clinical findings and natural history of PBC differ significantly among patients, ranging from asymptomatic with slow progression to symptomatic and rapidly evolving (94,95).

Clinical findings Although non-specific, fatigue is the most common symptom of PBC, up to 80% of PBC patients complain about chronic fatigue and more than 40% suffer moderate-to-severe symptoms (96,97). No correlation with the severity of liver disease has been demonstrated, however, fatigue can be associated with decreased overall survival (98,99). The mechanism of fatigue in PBC patients remains unknown, despite many proposals including autonomic dysfunction (100), muscle impairment (101), daytime somnolence (102), and altered central nervous system excitability (103,104).

PBC is more frequently associated with pruritus than other chronic cholestatic liver diseases. During the course of the disease, 20% to 70% of patients complain of pruritus as the most distressing symptom. It develops independently of the degree of cholestasis and the stage of the disease (105).

Bone density reduction is common in patients with PBC, with osteopenia (33%) and, less frequently, osteoporosis (11%) (106,107). Contrary to this, recent reports suggested that PBC might not represent an additional risk factor for bone demineralization in women with compensated disease when supplemented with calcium and vitamin D (108). Therefore, in clinical practice, such supplementation, along with monitoring of bone density and vitamin D, is highly recommended, even in patients at an early stage (89,90).

Several autoimmune diseases could coexist with PBC in up to a third of patients, most frequently with Sjögren’s syndrome and autoimmune thyroid disease. Coexistence of other autoimmune diseases does not modify the course or clinical manifestation of PBC, with the exception of a reported slower progression of liver fibrosis in patients with scleroderma (109,110).

Once PBC has progressed to cirrhosis, clinical features of liver dysfunction do not differ from those seen in patients with cirrhosis due to other causes, with the exception of esophageal varices, which can develop early in the disease course, sometimes before other signs of cirrhosis (111). The occurrence of hepatocellular carcinoma in PBC patients is similar to those with cirrhosis of other etiologies and warrants surveillance in patients at an advanced disease stage (112).

Natural history The natural history and prognosis of PBC has become notably benign with significantly decreased mortality within the last two decades (1,8,113). Although these observations could be secondary to early diagnosis (1,8) and a consequent lead-time bias (4), falling rate of liver transplantation for PBC in western countries since widespread use of UDCA suggest an actual change in the natural history of PBC (13,14).

Classically, PBC patients can progress from an asymptomatic stage to symptomatic stage with symptoms attributable to liver damage, such as itching, jaundice, esophageal varices, ascitis, and/or encephalopathy (1). The natural history of PBC has become more difficult to characterize given the rising number of asymptomatic cases which require long-term follow-up (113). Formerly, the presence of symptoms at diagnosis was an important determinant of disease progression and survival (114,115). The 10-year survival of asymptomatic patients ranged from 50 to 70%, whereas the median duration of survival for symptomatic patients ranged 5 to 8 years from the onset of symptoms (87,114-116). Additionally, asymptomatic PBC patients were shown to have shortened survival compared to a healthy population (87,116). However, in a study from the UK of a large cohort of patients followed up for 24 years, although mortality due to liver disease was greatest in symptomatic patients, overall survival was similar in individuals with and without symptoms at the time of presentation (95). It seems difficult to conclude the impact of the presence of symptoms in PBC patients at present, however, considering the prognostic relevance of the presence of symptoms is well documented, and the higher proportions of asymptomatic patients enrolled in the more recent cohort studies explain, at least, the observed improvement in the natural history of PBC since the 1980s (117).

Prediction of patients’ survival with PBC has been attempted, and the Mayo model is the most well regarded, which includes five independent variables (age, total bilirubin, albumin, prothrombin time, and severity of ascitis), with amount of bilirubin in serum as the most heavily weighed (118).

2.5. Treatment of PBC

The only currently established treatment for PBC is UDCA 13-15 mg/kg a day (119). UDCA was shown to improve serum biochemical markers such as bilirubin, ALP, GGT, cholesterol, and IgM levels (120-125).
UDCA may slow down histologic progression to liver cirrhosis (124,126), improve quality of life, survival free of transplant, and overall survival (9-12,127). However the mechanisms of UDCA in chronic cholestasis remain enigmatic (128), and about a third of patients are not sufficiently controlled with UDCA monotherapy, necessitating liver transplantation or an additional therapeutic approach (2).

**UDCA treatment for PBC-Natural history in the UDCA era** UDCA is currently considered as the mainstream for treatment for PBC (89,90). Mechanisms of action of UDCA remain unclear, yet the hydrophilic nature of it could lead to a reduction in amounts of bile acids, and it might also regulate cellular signaling and protect against apoptosis (129,130).

The rate of progression to cirrhosis was 13% in patients with UDCA and 49% in those without UDCA after a 6-year follow-up (131). Another study revealed that the rate of progression to cirrhosis was 7% in patients with UDCA and 34% in those without UDCA after a 4-year follow-up, with 76% of UDCA-treated patients remaining in an early disease stage, as compared to 29% of placebo-treated patients (126).

A combined analysis of four clinical trials including 367 patients also found significantly decreased disease progression in patients with UDCA (132). Another two also revealed improved survival without liver transplantation or reduction in the risk of death in patients with UDCA (127,133).

The risk of developing varices was 16% for UDCA-treated patients which was significantly lower than that of 58% for those receiving the placebo in a 4-year prospective observation (134). Additionally, many studies have demonstrated an improvement of biochemical and histological features with UDCA administration (120-125).

Recently four long-term trials have found improved survival in UDCA-treated patients (9-12). Corpechot et al. (9) reported in 262 patients who had received 13-15 mg/kg UDCA daily for a mean of 8 years that the overall survival rates were 84% and 66% at 10 and 20 years, respectively. In early-stage patients, predictions to progress to liver transplantation or death were 6% and 22% at 10 and 20 years, respectively. The survival of early-stage patients was similar to that of the control population. In contrast, the probability of death or liver transplantation was significantly increased in patients treated in late-stages. Three other studies (10-12) also reported similar results. The survival rate of patients in early stages who biochemically responded to therapy was similar to that of the control populations in these studies. "Response" here is defined as a decrease in ALP to < 40% (10), or serum bilirubin < 1 mg/dl, ALP < 3x upper limit of normal, and AST < 2x upper limit of normal (11,12). 10-year transplant-free survival of 90% in responders was reported, while it was 51% in non-responders (11).

Despite these encouraging results, the beneficial effects of UDCA on survival have been questioned repeatedly. Actually, among a number of randomized placebo-controlled studies on UDCA (121-125,135,136), none of them could find a survival benefit with UDCA, and only two studies of an extension follow-up found a positive effect on survival (137,138). Accordingly, several studies of meta-analysis based on these randomized studies concluded that UDCA has no benefit on the reduction of mortality and liver transplantation (139-141). The reported overall mortality and the rate of liver transplantation in PBC patients were both around 6% irrespective of the use of UDCA (140). However, when meta-analysis is performed with studies of a follow-up period over 2 years and with those using an effective dose of UDCA of more than 10 mg/kg/day, UDCA significantly improves quality of life and transplant-free survival, and delays histologic progression in early-stage patients (142,143). Current guidelines therefore recommend to treat PBC with UDCA using doses of 13 to 15 mg/kg/day and to start treatment early (89,90).

Considering the established autoimmune of the disease, corticosteroids and other immunosuppressive agents have been evaluated for therapeutic use in PBC. Prednisolone in combination with UDCA was proved to improve biochemical/histologic findings (144,145), however, not only the lack of survival benefit but also serious side-effects have precluded its use except for overlapping autoimmune hepatitis disease (146,147). Other immunosuppressive agents including budesonide (148), azathioprine (149), cyclosporine (150), mycophenolate mofetil (151), or methotrexate (152), and drugs with antifibrotic properties including penicillamine (153), colchicines (154), and silymarin (155) have been investigated, however, none of these drugs was shown to provide any additional benefit, in terms of clinically relevant events, when compared to UDCA monotherapy.

Currently, about two thirds of patients treated with UDCA according to guidelines respond adequately and may have a normal life expectancy, while in the remaining one third of patients who fail to achieve a biochemical response or who are at an advanced stage at presentation, therapeutic options are extremely limited (156) and liver transplantation is necessitated as a last resort for cure (13).

### 3. Liver transplantation for PBC

PBC represents a major indication for liver transplantation in western countries, and to date over 6,000 liver transplants have been performed for PBC in the US and Europe (16,157). Over time, however, a notable decline in the number of liver transplants and waiting list additions for PBC has been observed (13,14). Data from United Network for Organ Sharing (UNOS)
show that the absolute number of liver transplantations increased by a mean 249 per year between 1995 and 2006, while the absolute number of liver transplantation performed for PBC decreased steadily by a mean of 5.4 cases per year in the same period (13). This is the case in Europe where a five-fold reduction of liver transplantation for PBC has occurred from 1988 to 2006 (158). Despite the decrease in number of transplant cases among increasing numbers of PBC patients, liver transplantation still remains as a last curable option for end-stage PBC patients.

3.1. Indication and timing of liver transplantation for PBC

Indications for liver transplantation in individuals with PBC do not differ from those in patients with other liver diseases, namely decompensated cirrhosis with intractable ascites and spontaneous bacterial peritonitis, recurrent variceal bleeding, encephalopathy, or hepatocellular carcinoma (90). Christensen et al. (159) demonstrated that the optimal timing for liver transplantation in PBC (defined as the point when the probability of survival after transplantation is greater than the probability of survival without transplantation) is when the serum total bilirubin reaches around 10 mg/dl. It is recommended to refer to the liver transplant unit when the total bilirubin reaches 6 mg/dl, the Mayo risk score is over 7.8, and the MELD score is over 12 (90).

Treatment-resistant pruritus, severe recurrent encephalopathy, and recurrent variceal bleeding (despite preserved liver function) may also merit consideration for liver transplantation (160). In selected cases with severe pruritus that is refractory to medical treatment, liver transplantation remains the last resort (18). On the contrary, fatigue, one of the principle factors contributing to impairment of the quality of life, is not an indication for transplantation as available evidence does not support the efficacy of the procedure (161).

3.2. Survival after liver transplantation for PBC

Patients with PBC have more favorable outcomes after liver transplantation than those with viral hepatitis or alcoholic associated disease (162). An analysis of data from the ELTR in 2003 revealed a 1-, 5-, 10-year patient and graft survival of 83, 77 and 69%, and 79, 71 and 64%, respectively, among 2,959 patients (163). Kashyap et al. (164) reported a retrospective analysis of the UNOS database of liver transplantation for PBC patients, in which a 1-, 3-, and 5-year patient survival among living donor liver transplantation (LDLT) and deceased donor liver transplantation (DDLT) was 93, 90 and 86 %, and 90, 87 and 85%, respectively, while a 1-, 3-, and 5-year graft survival among LDLT and DDLT was 86, 81 and 77%, and 85, 83 and 81%, respectively. A Japanese study investigating LDLT for PBC revealed 1- and 5-year survival of 80% and 75%, respectively (165). With excellent survival, recurrent disease has become a high-lightened problem in recipients who had received liver transplantation for PBC.

3.3. Recurrent PBC after liver transplantation

Recurrent PBC after liver transplantation was first reported in 1982 (166). Despite initial controversy, the recognition of recurrent PBC is now firmly established in the liver transplant community (16). Recent studies (14,167-181) of liver transplantation for PBC in individual programs (with over 40 PBC transplant cases) are summarized in Table I. Although most studies at present have concluded that recurrent PBC has little impact on patient or graft survival following liver transplantation in the short- and medium-term, taking into account a considerable number of patients developing recurrent disease, continued longer follow-up may identify impaired long-term patient/graft survival in patients with recurrent PBC. Ongoing debates for recurrent PBC focus on defining the factors associated with recurrent PBC so that strategies for prevention and treatment can be established. Additionally, investigation of recurrent PBC after liver transplantation may also provide helpful insights into the pathophysiology of PBC in the native liver.

3.4. Diagnosis of recurrent PBC

Unlike PBC in the native liver, clinical manifestations of recurrent PBC is not specific. Pruritis and jaundice, typical disease-related symptoms in PBC are rarely encountered in recurrent PBC. Fatigue, the most common complaint in PBC, and metabolic bone disease can be multifactorial and remain nonspecific in post-transplant patients (16). Disease-related symptoms in recurrent PBC have been reported in about 10% patients in previous reports, with fatigue and pruritis being the most common complaints as in pre-transplant PBC (173,174). Yet, most studies do not address disease-related symptoms, which might represent the rarity and difficult recognition of manifestations of recurrent PBC.

Similarly, most patients with recurrent PBC have normal or clinically insignificant elevation of serum liver biochemistry tests at the time of diagnosis (169,174,182,183), unlike at the diagnosis of PBC with native liver. Serum AMA, the most important criterion for the diagnosis of PBC, is not a marker for recurrence (182,184). The persistence of serum AMA has been demonstrated in previous studies, with immediate reduction after liver transplantation and subsequent identification in postoperative serial investigations (175,185-187). There seems to be no correlation between the presence or titer of serum AMA and the development of recurrent disease (182).
The gold standard for the diagnosis of recurrent PBC is histologic confirmation (188). The histologic hallmark of recurrent PBC is granulomatous cholangitis or the florid duct lesion (167, 170-172, 183, 184, 186, 189), which are present in approximately 60% of initial diagnostic liver biopsies (174). Less frequent inflammatory features, such as dense lymphoplasmacytic or plasma cell infiltrates within the portal tract, may correlate with subsequent disease recurrence (167, 183, 190). At the same time, it is important to differentiate between recurrent disease and other causes of bile duct damage in the graft, such as acute or chronic allograft rejection, ischemic injury, infection, or drug damage, though it may often be difficult or indeed impossible. The diagnostic criteria advocated by Neuberger et al. are presented in Table 2 (188).

### 3.5. Epidemiology of recurrent PBC

The reported prevalence rates range from 1-35% in recent studies (Table 1). This wide variety may be due to different diagnostic criteria, the use of protocol biopsies by transplant units, and difficulty in histologic diagnosis. The most important factor relates to the use and timing of liver biopsies in follow-up (190), meaning performing biopsies for clinical indications alone will underestimate the prevalence rate (16).

The time to recurrence also varies between studies, as shown in Table 1. The serial investigation at the same centers have reported increasing rates of recurrent disease over time indicating that more cases would be identified with a longer follow-up period (14, 174, 182, 191). Among centers with over 100 cases of PBC transplants with long follow-up, the time to recurrence, and 10- and 15-year cumulative incidence rate were reported to range from 3.5-5.8 years, and 21-37% and 43%, respectively (14, 172, 174, 176, 178, 179).

### 3.6. Factors associated with recurrence

As described in the previous section, there is increasing evidence for a genetic predisposition for developing PBC in native liver. Accordingly genetic predominance, especially, the effect of HLA matching between donor and recipient, has been investigated in the disease recurrence of PBC patients after liver transplantation, however, it remains controversial so far. HLA allele frequency analysis by Sanchez et al. (169) demonstrated that patients with recurrence did have significant allele similarities; the donor alleles A1, B57, B58, DR44, DR57 and DR58 and the recipient allele B48 were found more often in recurrent PBC. Morioka et al. (175) suggested that a lower number of HLA mismatches between donor and recipient was an independent risk factor for disease recurrence following LDLT. Similarly, two large studies of DDLT reported...
of both recipient and donor could be a debate in the future, considering the increasing number of PBC patients receiving liver transplants at older ages and the increasing use of older deceased donor livers, although the available studies at present are conflicting (14,174). Few studies investigated graft quality, suggesting that poor qualities of graft such as ischemic time (14,174) and graft histology (fibrosis and steatosis) (177) were risk factors for disease recurrence.

### 3.7. Disease progression and treatment

As shown in Table 1, graft or patient loss due to progression of recurrent disease is extremely rare, representing no effect on long-term outcomes in all studies. Two leading institutes of liver transplantation, the Mayo and Birmingham groups, have reported 3 out of 485 and 2 out of 154 cases required retransplantation, respectively (172,174). The Mayo group also reported the result of sequential protocol biopsies of definitely recurrent PBC, revealing that periportal fibrosis was present in 8 of 17 (47%) with a mean follow-up of 5.9 years and that 2 of them further developed septal fibrosis during an additional 3 years of follow-up (182).

The Birmingham group recently reported that the rate of graft loss due to recurrent disease was 5.4% among 541 liver transplants for PBC with a median time from the diagnosis of recurrence to graft loss of 7.8 years, which was significantly lower than those of other etiologies (199). With a longer follow-up, recurrence of disease could have an impact on patient and graft survival.

To date no standard guideline exists for treatment of recurrent PBC. The modification of immunosuppression has not been formally reported as an interventional study. Based on the aforementioned possible beneficial effect of UDCA and its widespread use in PBC patients, and given nearly all recurrent PBC is diagnosed at an early stage, many authors have pointed out the potential role of UDCA for recurrent PBC after liver transplantation (14,169,170,174,178). However, in addition to the limitation of the small number of recurrent disease cases, normal or near normal serum liver biochemistries at initial diagnosis of most recurrent patients makes it challenging to assess the efficacy of UDCA. A recent report from the Mayo group found that 52% of patients with recurrent PBC treated with UDCA showed normalization of liver biochemistries, while the liver biochemistry normalization rate was 22% in untreated patients. However there was no significant difference in histologic progression between those with and without UDCA. Moreover, no long-term survival benefit was observed in patients treated with UDCA in the study (174). Whether UDCA treatment has an impact on the natural history of recurrent PBC needs to be further investigated in controlled trials with extended follow-up.

### Table 2. Diagnostic criteria for recurrent PBC

| 1. Liver transplant for well-described PBC |
| 2. AMA seropositive after liver transplant |
| 3. Liver histology with the following characteristics |
| a) Mononuclear inflammatory infiltrate |
| b) Lymphoid aggregates |
| c) Epithelioid granuloma formation |
| d) Bile duct destruction |

Definite recurrent PBC: 3 of 4 portal tract lesions are observed
Probable recurrent PBC: 2 of 4 portal tract lesions are observed

Abbreviations: AMA, antimitochondrial antibody; PBC, primary biliary cirrhosis.
4. Conclusion
The clinical course of PBC has become notably benign with the use of UDCA at an early stage and the establishment of liver transplantation for end-stage disease. In addition to investigations for pathogenesis of this entity and new treatment for those without response to UDCA therapy, the following issues need randomized controlled trials; the actual survival benefit of UDCA in the natural course of PBC, and the impact of cyclosporine and UDCA for those with recurrent disease after liver transplantation.

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(Received March 23, 2012; Revised April 28, 2012; Accepted May 11, 2012)
Serum microRNA is a promising biomarker for osteogenesis imperfecta

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1. Introduction

Osteogenesis imperfecta (OI) is a rare hereditary disease characterized by abnormality of osteoblastic proliferation and differentiation and osteoclastic activity (1). Emerging evidence has suggested that microRNAs (miRNAs) may be important mediators of ossification through binding and repressing the expression of target mRNAs whose translation products are involved in osteoblastic proliferation and differentiation and osteoclastic activity (2).

miRNA is a class of small noncoding RNAs that principally function in the spatiotemporal regulation of protein translation by binding to the 3’-untranslated region (UTR) of target mRNAs, leading to mRNA degradation or translational repression (3). Since discovered in the early 1990s, miRNAs have been shown to play important regulatory roles in a wide range of biological and pathological processes (4-6). As an existing form,
serum miRNAs are thought to be a greatly promising novel biomarker for cancers, nerve-development diseases, and some metabolic diseases with their high accuracy (sensitivity and specificity) and predictability (7). However, little attention has been devoted to the relationship between serum miRNAs expression and bone-related diseases.

To learn more about the potential relevance of serum miRNAs in bone-related diseases, we screened more than 100 bone-related miRNAs in serum samples from patients with OI and healthy controls to find OI-related miRNAs, and with bioinformation analysis, we examined whether these differential bone-related miRNAs have potential to be markers for OI diagnosis.

2. Materials and Methods

2.1. Serum collection and storage

The study protocol was approved by the Ethical Committee of Shandong Medicinal Biotechnology Center, Ji’nan, Shandong, China. Whole blood samples were collected from 22 osteogenesis imperfecta patients (age, 9.54 ± 3.65 year) and 10 healthy donors (age, 8.20 ± 0.42 year). Serum was isolated by centrifuging whole blood at 1,900 × g for 10 min at room temperature. To minimize degradation, samples were processed within 2 h. Twenty-two OI patients came from Tianjin Hospital and Shandong Provincial Hospital with patient’s informed consent, and 10 healthy controls came from volunteers in Ji’nan.

2.2. Total RNA isolation

Total RNA from serum was isolated using TRIzol LS (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. In brief, 200 μL serum was mixed with 750 μL of TRIzol LS, and then 200 μL chloroform was added, the samples were shaken vigorously for 30 sec, and allowed to stand for 5 min at room temperature. After centrifugation at 12,000 × g for 15 min at 4°C, the upper aqueous phase was transferred to a fresh tube and 600 μL isopropyl alcohol was added followed by mild mixing and incubation for 5 min at room temperature. After centrifugation at 12,000 × g for 10 min at 4°C, the upper aqueous phase was discarded and the precipitate was washed twice with 750 μL of 75% ethanol. Finally, RNA was diluted in a final volume of 15 μL RNase-free water. The RNA integrity and concentration were determined using 2% agarose gel electrophoresis and a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Quantification of serum miRNAs by real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

The first-strand miRNA-cDNA PCR template was generated from ~50 ng of total RNA using a miRNA first-strand cDNA synthesis kit (Tiangen, Beijing, China) according to the manufacturer’s instructions. Then approximate 2.5 ng of cDNA was used to amplify using a miRcute miRNA qPCR detection kit (Tiangen) with a LightCycler 480 Sequence Detection System (Roche Applied Science, Mannheim, Germany). In the qRT-PCR, amplification was carried out as follows: 94°C for 2 min, 45 cycles of 94°C for 20 sec, 60°C for 20 sec, and 72°C for 20 sec. Data were generated from three independent qRT-PCR reactions and the relative quantity of miRNA expression was calculated using the comparative cycle threshold (Ct) method, in which the lowest expressed sample was used as a calibrator. Subsequently, the relative quantity was normalized using geometric averaging of selected reference genes.

2.4. Selection of reference genes

Six OI patients and 2 healthy controls with different characteristics (gender, age, and medication history) were selected as the objects for this study. In the selected 6 candidate reference genes (miR-16, miR-92a, miR-638, Let-7a, snRNAU6, and 18S rRNA), miR-16, miR-638, snRNAU6, and 18S rRNA have been regarded as common internal references (8), and miR-92a and Let-7a were two miRNAs which were stably expressed in our previous trials. Using qRT-PCR followed by geNorm software analysis, which was used to sort gene expression stability and find optimal reference gene(s) (9), the expression stability of 6 candidate reference genes was obtained and an optimal number of reference genes for normalization was determined. For verifying accuracy and universality of chosen reference genes, stable expression, validation was carried out by means of normFinder (10), Bestkeeper (11), and delta CT method analyses (12) and increasing sample size. Six candidate reference genes’ primer sequences were as follows: for hsa-miR-16, 5'-TAG-CAG-CAC-GTA-AAT-ATT-GGC-G-3'; for hsa-miR-92a, 5'-TAT-TGC-ACT-TGT-CCC-GGC-CTG-T-3'; for hsa-miR-638, 5'-GGG-TGG-CGG-CCT-AAA-AA-3'; for hsa-Let-7a, 5'-TTG-AGG-TAG-TAG-GTT-GTA-TAG-TT-3'; for snRNAU6, 5'-TGC-GGG-TGC-TCG-CTT-CGG-CAG-C-3'; for 18S rRNA, 5'-CAG-CCA-CCC-GAG-ATT-GAG-CA-3'.

2.5. Screening of differential expression of bone-related miRNAs in serum of patients with OI

Through three miRNAs target genes analysis softwares, miRanda, Targetscan, and Pictar, we sorted out some miRNAs which could combine complementarily to miRNA 3’ UTR of genes functioning in osteogenesis. Together with miRNAs verified to regulate the course of bone formation, bone-related miRNAs have been chosen, and these miRNAs were quantified in serum of 8 OI patients without medication history and 8 age-matched
healthy controls using qRT-PCR with multiple reference genes as endogenous controls. Finally, a matched t test was used to screen out the differential expression bone-related miRNAs.

3. Results

3.1. snRNAU6, miR-92a, miR-16, and Let-7a as internal references for qRT-PCR normalization

Based on that the expression ratio of two suitable reference genes should be identical in all samples regardless of the experimental conditions, we utilized geNorm software to assess the expression stability of 6 candidate control genes and found all 6 candidate reference genes had a stable expression level in serum of 8 healthy controls and 8 patients with different characteristics (M < 1.5, the system default value is 1.5). The order of their expression stability is snRNAU6/miR-92a > miR-16 > Let-7a > 18S rRNA > miR-638 (Figure 1). With pairwise variation analysis, the optimal number of reference genes for normalization was determined to be 4 (V_{c5} = 0.133 < 0.15, the system default value is 0.15) (Figure 2). Further validation by means of normFinder, Bestkeeper, and delta CT method analyses and increasing sample size revealed the same results (Figure 3), which revealed that snRNAU6, miR-92a, miR-16, and Let-7a could be used as an internal reference group for qRT-PCR normalization.

3.2. Differential expression of bone-related miRNAs

In the more than 100 bone-related miRNAs we have chosen with the help of software forecasts and literature reports, miR-26a, miR-30e, and miR-21 were revealed to be up-expressed and miR-34c, miR-29a, miR-29b, miR-489, miR-133a, miR-145, miR-210, and miR-1297 down-expressed in serum of OI patients compared to healthy controls (p < 0.05) (Figure 4). In the differential expression, miRNAs, miR-29a, and miR-133a were significantly down-regulated, at 12.80-fold and 6.31-fold, respectively. Compared to the healthy control group, the quantity of miR-29a had a universal lower level in the patient group, and on the contrary, miR-26a had a universal upper level.

4. Discussion

Although real-time reverse transcriptase PCR has become the technology of choice for high-throughput and accurate expression profiling of target genes, the selection of a proper internal reference was still a festering issue. Unconditional stable expression housekeeping genes are not known to exist in nature, and will lead to large errors.
with normalization based on a single reference gene (9). Therefore, we inputted geNorm software to seek out more stable genes as an internal reference group, and the qRT-PCR data was then normalized using a geometric mean of selected genes. In 4 selected reference genes, miR-16 was the most common reference for miRNAs detection in peripheral blood of various diseases, such as gastric cancer (8), prostate cancer (13), breast cancer (14), and liver cancer (15). Another gene, snRNAU6, a kind of intracellular stable expression ribosomal RNA, recently has been found to express stably in the peripheral circulation of diverse diseases, for instance, in the plasma of colorectal and oral squamous cell carcinoma, in the serum of breast cancer and in the urine of bladder cancer (15).

Most alternatively expressed bone-related miRNAs we observed have been regarded as potential biomarkers for various cancers; for instance, miR-21 was called "oncogenic miRNA", precisely because it had abnormal expression in many cancers (16-21). Although no report directly indicated that there is a close relationship between differential expression of miRNAs and bone-related diseases, these miRNAs have been verified to be involved in the course of ossification (2). While investigating the relationship between bone morphogenetic proteins (BMPs) and miRNAs in phenotypic induction in osteoprogenitors, Li Z et al. found that miR-133 functionally inhibits osteoprogenitor differentiation by attenuating Runx2 and Smad5 pathways, which synergistically contribute to bone formation (22). miR-29a has been reported to suppress expression of Dkk1, Kremen2, and sFRP2, all of which are negative regulators of Wnt signaling, which promotes osteoblast differentiation (23). In a study to determine miR-29b function, transforming growth factor beta 3 (TGFβ3) was found to inhibit osteoblast differentiation, while miR-29b was observed to promote osteoblastogenesis by directly targeting the 3' UTR sequence of TGFβ3 mRNA (24). In a similar report, miR-210 was found to enhance osteoblastogenesis by inhibiting the TGFβ/activin signaling pathway through targeting and repressing translational products of the AcvR1b gene (25). miR-21, regulated by the RANK ligand (RANKL)-induced c-Fos, a critical transcription factor for osteoclastogenesis (26), conversely promotes c-Fos expression by downregulation of programmed cell death 4 (PDCD4) protein levels, due to repression removal from c-Fos (27). Therefore, a positive feedback is formed between miR-21 and c-Fos, which significantly enhances the regulation capability of miR-21 for osteoclast differentiation.

Although our findings demonstrate the potential utility of altered expression of serum miRNAs as OI biomarkers, it is necessary to acknowledge that the serum sample size of OI patients observed in this study was relatively small. Nonetheless, these data suggest that serum miRNAs could be a promising biomarker for diverse diseases.

In conclusion, the discovery of altered expression of bone-related miRNAs in serum of OI patients has opened up new possibilities for bone-related disease diagnosis and expressed serum miRNAs are a promising biomarker for osteogenesis imperfecta.

Acknowledgements

This work was supported by a grant from National Key Technology R&D Program (No. SQ2011SF12C03081).

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Figure 4. Differential expression levels of eleven serum miRNAs in healthy controls and patients with OI. Data were obtained from quantitative detection of bone-related miRNAs in serum of OI patients. Closed columns, healthy controls; open columns, patients.


(Received March 23, 2012; Revised April 20, 2012; Accepted April 23, 2012)
Accessory lobes of the liver: A report of 3 cases and review of the literature

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1. Introduction

Accessory lobe of the liver (ALL) is congenital ectopic hepatic tissue mostly due to embryonic heteroplasia, though in rare instances an ALL may occur after trauma or surgery. There are two types of ALL: an accessory lobe joined to normal hepatic tissue and a lobe that is completely separate (1). An ALL, and especially a completely separate ALL, is rarely clinically and is difficult to diagnose before surgery, so it is easily missed or misdiagnosed. Hundal noted that from 1925 to 2006 there were 18 cases of an ALL diagnosed after surgery or biopsy (2). The current authors report 3 additional cases that were positive diagnosed by computed tomography (CT) and magnetic resonance imaging (MRI) and confirmed by surgical and histological examination. The pertinent literature on accessory lobes of the liver is also reviewed.

2. Case report

2.1. Case One

Chest films for a 59-year-old female showed a mass in the lower right costophrenic angle with distinct margins and a uniform density. The mass was thought to be a benign tumor in the pulmonary basal segment. An ultrasound was performed to differentiate the mass from a subphrenic mass. The exam showed a lesion occupying the right posterior lobe of the liver with indistinct margins, a uniform echo, and blood flow. This was thought to be a benign tumor possibly originating from the liver. Unenhanced MRI scan: T1-weighted imaging (T1WI) showed an irregular mass in the right posterior lobe of the liver, all of its margins were distinct except the inboard part. The mass had a homogeneous signal (Figure 1A). T2-weighted imaging (T2WI) showed that the mass had the same signal intensity as normal liver tissue and a homogenous signal; vascular opacities were apparent inside (Figure 1B). Coronary scanning showed distinct margins and a low signal line demarcating the mass from normal liver tissue (Figure 1C). Vertical scanning showed a mass connected to the right lobe by a stalk of tissue (Figure 1D). MRI diagnosis: Pedunculated ALL.

2.2. Case Two

A 20-year-old male visited the hospital due to coughing and a fever. Plain films showed an irregular mass in the right lower lung field; its margins were distinct and it had a uniform density, and there was a clear line demarcating it and normal liver tissue (Figure 2A). Enhanced scanning showed that the mass had the same degree of contrast enhancement as normal liver tissue.
and a uniform density. Hepatic veins were connected to the inferior vena cava (Figure 2B). Delayed phase: The mass was demarcated from normal liver tissue by an enhanced line (Figure 2C). CT diagnosis: Completely separate ALL.

2.3. Case Three

A 9-year-old female was brought to the hospital due to coughing, expectoration, and a fever lasting 3 days. The clinical diagnosis was pulmonary infection. Plain films showed a lesion occupying the right lower lung field; its density was uniform and most of its margins were distinct except at the surface of the diaphragm. T2WI showed a quasi-circular mass in the right posterior lobe of the liver; its margins were distinct and its signal intensity was homogeneous and the same as that of the right lobe of the liver (Figure 3A). Coronary scanning showed a mass located at the rear of the right lobe with its base connected to the right lobe and a continuous vascular structure inside (Figure 3B). Sagittal scanning showed that the mass connected to the right lobe via a wide base, and vessels heading to the right lobe were apparent (Figure 3C). MRI diagnosis: Sessile ALL.

3. Discussion

3.1. The mechanism of ALL

Meckel reported several cases of ALLs (3). An ALL is an anatomical abnormality that is rarely seen and is mostly the result of embryonic heteroplasia (4,5), though in rare instances an ALL may occur after trauma or surgery (6). As a type of congenital anatomical malformation, ALL occurs very rarely because it is associated with an autosomal recessive gene with a very low frequency. Anatomical research through necropsies of 172 rats confirmed this genetic theory (8). Currently, there are two hypotheses of the mechanism of an ALL: (i) the embryonic liver curls outwards and forms an accessory lobe during the embryonic stage of development (10) or (ii) an accessory lobe arises from intra-abdominal hypertension caused by the development of the tunica muscularis recti and the enlargement of the liver (11).
normal liver tissue that is most often seen in the thorax or pelvic cavity (4,13,14); or (iv) a pinpoint atopic ALL (< 10 g) that is most often located at the margins of the liver or even gallbladder wall. An abdominal ALL, and especially a right abdominal ALL, is reported relatively frequently. There are few reported cases of an ALL located in the thorax or pelvic cavity (5,15,16). In the 3 cases reported here, the ALL was connected to normal liver tissue by an enhanced line.

3.2. Classification of ALL

There is little literature about the classification of ALL. ALL can be classified into two types according to Stattaus (1): an accessory lobe joined to normal hepatic tissue or a completely separate accessory lobe. An ALL can also be classified as pedunculated or sessile. There are several types of ALL that are classified by volume and weight (4,6,12); (i) a bulky ALL (> 31 g) connected to the liver via a stalk of tissue or wide base in the subphrenic or perihpatic zone; (ii) a small ALL (11-30 g) connected to the liver via a wide base on the surface of the liver or around the right posterior lobe; (iii) a completely separate ALL with no connection to normal liver tissue that is most often seen in the thorax or pelvic cavity (4,13,14); or (iv) a pinpoint atopic ALL (< 10 g) that is most often located at the margins of the liver or even gallbladder wall. An abdominal ALL, and especially a right abdominal ALL, is reported relatively frequently. There are few reported cases of an ALL located in the thorax or pelvic cavity (5,15,16). In the 3 cases reported here, the ALL was connected to normal liver tissue: 1 case involved a pedunculated ALL while
2 involved a sessile ALL. Classified by volume and weight, 1 case involved a bulky ALL while 2 involved a small ALL. All of the accessory lobes were located at the posterior costophrenic angle and on the surface of the liver.

3.3. Clinical features

Most patients with an ALL have no symptoms and are seldom diagnosed in the early stages. Most ALLs are discovered unexpectedly during surgery or autopsy. The rate of ALL detection is increasing thanks to advances in imaging equipment and the greater prevalence of physical examinations \((5,15,16)\). Patients with an ALL and no complications have no symptoms or physical signs but may occasionally present with acute stomachaches, recurring stomachaches \((1)\), precordial pain, nausea, or vomiting \((17)\). Though these problems are worse than the dyspnea caused by an ALL in the thorax \((18)\), they are not specific indicators of an ALL. The clinical manifestations of an ALL depend on complications, such as torsion of an ALL, traumatic rupture, or infarction \((6)\). Torsion of an ALL is most frequent and severe complication. Most patients with torsion of an ALL visit the hospital complaining of a severe stomachache due to hemadostenosis, vascular obstruction, ischemia, putrescence, or even rupture and bleeding \((19-21)\). During the 7th and the 8th week of embryo development, which is when the muscular layer of the abdominal wall is formed, development of an ALL in the embryo may obstruct the closing of the umbilical ring \((3,10)\), which is why most ALLs are associated with acromphalus. ALLs are also associated with congenital biliary atresia \((22)\), congenital diaphragmatic defects, and angiocavernoma \((8)\). Most reported cases of an ALL involve females ranging in age from newborns to 75 years. One of the current cases involved a male while the other two involved females. The male was age 20 while the females were age 9 and 59. One was diagnosed with an ALL based on a CT scan after a physical examination while the other two were diagnosed during a CT/MRI scan.

3.4. The diagnosis of ALL

ALL is rarely seen clinically and is difficult to diagnose before surgery \((2)\). It is often misdiagnosed because most patients with an ALL have no symptoms \((8)\). Many cases of an ALL were mis-diagnosed as an intraperitoneal tumor, pulmonary tumor, or diaphragm tumor \((23-25)\). The position of an ALL varies in each individual. An ALL is typically detected and diagnosed via plain films, contrast enhancement, radioactive species, type-B ultrasound, CT, or MRI \((5,26-28)\). Most previous cases of ALL were diagnosed by pathology after surgery \((23,26,29)\), though a few were diagnosed by imaging before surgery \((4,27,28,30)\). Rapid advances in medical imaging equipment such as that used for ultrasound, CT, MRI, PET, and especially multi-slice spiral CT (MSCT) and MRI multiplane imaging provides more accurate information for diagnosis of an ALL, including its size, shape, classification, position, and blood supply. Thus, an increasing number of patients with ALL can be accurately diagnosed in the early stages or before surgery \((5,31,32)\). A look at the CT and MRI findings from the 3 current cases indicates that: (i) according to CT/MRI scanning and enhanced scanning, the substantive part of the accessory lobe had the same density or signal as normal liver tissue; (ii) the ALL had distinct and smooth margins, with complete demarcation; (iii) the ALL was connected to normal liver tissue via a stalk of tissue or base; (iv) flowing-void vascular imaging was apparent during MRI scanning and venous imaging was apparent during enhanced CT/MRI scanning. An ALL connected to normal liver tissue can be accurately diagnosed based on these findings, though diagnosing a completely separate ALL is difficult since it cannot be readily differentiated from a mediastinal neoplasm or peripheral tumor.

3.5. Differential diagnosis

An ALL connected to normal liver tissue can be readily diagnosed based on CT/MRI characteristics though diagnosis of a completely separate ALL is difficult. In order to make an accurate diagnosis, factors such as size, shape, position, whether complications are present or not, and the type of complication should be considered. In terms of position, (i) an ALL in the thorax should be differentiated from a tumor of the pleura, lungs, chest wall, or diaphragm \((1,4,24,26)\) while (ii) an ALL in the pelvic cavity should be differentiated from a benign or malignant tumor of the pelvic organs \((33)\) and (iii) an ALL on the surface of the liver or abdominal organs should be differentiated from pathological changes in the liver, gall bladder, pancreas, spleen, or adrenal glands \((13,22)\). Most patients with an ALL have no clinical manifestations and the ALL may be large enough to jostle surrounding organs. This is completely distinct from an invading malignant tumor that destroys surrounding tissue and metastasizes to distant organs. Differentiating a completely separate ALL from a benign tumor is difficult without a pathological examination after surgery.

Patients with a pedunculated ALL may suffer acute stomachaches, abdominal distension, nausea, vomiting, or shock while they have complications of reversion, hemorrhage, or infarction \((5,6,25,28,34-38)\). Since such an ALL can easily be mistaken for an acute abdomen or abdominal tumor, CT or MRI imaging should facilitate differentiation.
3.6. Complications

Patients with an ALL may have overt symptoms or their lives may be in danger if they have complications (39). Reported complications include reversion, infarction, hemorrhage, fracture, hemangioma, biliary atresia, gallbladder torsion, hepatic dysfunction, diaphragm defects, and acromphalus (40). Whether complications are present or not and the type of complication relate to the patient’s age and the type, size, and position of the ALL. Most infants with an ALL also have congenital acromphalus, congenital biliary atresia, or gallbladder torsion (22,41). A pedunculated ALL carries a higher incidence of reversion than the other types of ALLs. Severe congestion and blood stasis result from an insufficient blood supply, vastly increasing the incidence of vascular ruptures (10,21). In cases of an ALL, one complication is often joined by others, just as reversion of an ALL occurs concurrently with infarction and rupture (17,20). Complications of an ALL can be classified as acute or chronic complications; the former are caused by strenuous exercise or trauma (6,28,38) while the latter, such as persistent hepatic dysfunction (40), do not appear in the early stages but do present as the ALL develops.

3.7. Treatment and prognosis

There is no need to treat patients with an ALL who have no symptoms or complications (13). A liver transplant or resection of the ALL should be performed for patients with serious complication at birth such as acromphalus, biliary atresia, or gallbladder torsion (42,43). Resection of the ALL should be performed in adults with serious complications. Patients are reported to have a satisfactory prognosis. There is no need to treat patients with a sessile ALL connected to normal liver tissue or completely separate ALL if they have no symptoms or complications. If a patient is diagnosed with a pedunculated ALL, surgery should be performed as soon as possible in order to avoid unexpected complications.

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26. Kostov DV, Kobakov GL. Accessory hepatic lobe. Surg...
Asperger's syndrome with unusual cerebral pathology: Case report and literature review

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Summary
A case of Asperger's syndrome with unusual cerebral pathological changes is reported. A 22-year-old male had been having diagnostic Asperger's syndrome since the age of eight and had epilepsy during the past two years. Radiological studies revealed a focal intra-axial cortical and subcortical cerebral lesion with hyper-intensity and non-enhancing contrast in the left frontal lobe. Histological and immunohistochemical studies demonstrated that the lesion consisted of cortical laminar disorganization, neuronal dysmorphism and increased heterotopic neurons in sub-cortical white matter. To our knowledge, this is the first case of Asperger's syndrome with focal cerebral pathological abnormalities rather than minicolumnar changes and the gyral malformation reported in the literature.

Keywords: Asperger's syndrome, brain, neuropathology

1. Introduction

Asperger's syndrome is characterized by impaired social communication with normal language skills and intelligence. Although this syndrome is not uncommon in childhood, the information about its neuropathological changes is very much limited, which has been barring the understanding of the etiology and pathogenesis of this disorder. The neuropathological findings that have been so far reported are cerebral cortical minicolumnar abnormalities and gyral malformation in four patients (1,2). Our report documents a case of Asperger's syndrome with cerebral abnormalities different from the previously reported neuropathological changes.

2. Case report

2.1. Clinical history

A 22-year-old male with history of Asperger's syndrome presented with chronic epilepsy for the past two years. The diagnosis of Asperger's syndrome was made when the patient was eight years old. For the past two years, the patient also had been having superimposed episodes of unusual movements, staring spells and headaches. The neuroradiological examinations showed a focal lesion with hyper-intensity and non-enhancing contrast in the cortex and subcortical white matter of the left frontal lobe, corresponding to Brodmann's area 47 (Figure 1). Surgical excision was subsequently performed, and the specimen was sent for pathological evaluation.

2.2. Macroscopy and microscopy

The excised brain specimen was grossly examined after fixation in 10% buffered formalin. The tissue was then paraffin-embedded, and 4-µm-thick sections were cut for staining. Sections were stained with hematoxylin and eosin as well as the avidin-biotin-complex immunoperoxidase technique with antibodies against glial fibrillary acidic protein (GFAP), neuron specific enolase (NSE), synaptophysin and Ki67. The stained sections were reviewed by three neuropathologists.

The specimen was received as soft tan and gray tissue fragments without hemorrhage and necrosis. Microscopically, the cerebral cortex and subcortical white matter were identified. The cortex showed...
laminar disorganization and scattered dysmorphic neurons, more significant in cortical layers 3, 4, 5 and 6 (Figure 2A). There were scattered dysmorphic or enlarged neurons in the white matter, mostly located in the subcortical region. The number of neurons and glial cells of cortex and glial cells of white matter was within normal range. The glial cell composition was unremarkable. There was no inflammation, neoplasm or hemorrhage in the specimen. Immunohistochemical studies demonstrated that clusters of GFAP positive glial cells and neuropile were distributed in an irregular and random fashion (Figure 2B). NSE and synaptophysin stains highlighted scattered dysmorphic neurons located in the cortex and subcortical white matter (Figure 2C). The proliferative index indicated by Ki67 was close to zero.

3. Discussion

Asperger’s syndrome is characterized by severe and sustained impairment in social interaction and odd or eccentric behaviors. The patient is often pre-occupied with complex thoughts. However, the patient usually has normal intelligence and adequate language skills in some aspects such as vocabulary and grammar. Clinical manifestation is pervasive. The diagnosis is usually made when the patient reaches school age.

Although this disorder is clinically chronic and significant, its neuropathological changes are barely known. The only reported pathological findings in the medical literature were alteration of total number and size of cerebral cortical mini-columns in two patients (1), macrogyria in one patient and polymicrogyria in another patient (2). Our case demonstrates another group of histopathological changes that can be classified as a cerebral developmental abnormality. The pathological changes in this case are morphologically similar to those found in focal cortical dysplasia and are
more severe than the pathological findings previously reported in Asperger's syndrome. Clinically, this patient developed epilepsy at an older age. Epilepsy is an essential manifestation in patients with focal cortical dysplasia. This phenotypic combination of Asperger's syndrome and epilepsy indicates that Asperger's syndrome and focal cortical dysplasia might share the same spectrum of developmental neuropathology. The lower end of this neuropathological spectrum such as changes of cerebral cortical mini-columns produces Asperger's syndrome without seizures, and the higher end of the spectrum like focal cortical dysplasia causes epilepsy, while the neuropathological abnormalities between these two extremes might clinically manifest as Asperger's syndrome with or without epilepsy. Our case initially had Asperger's syndrome at a younger age and developed superimposed seizures at a later age. It is possible that the current cerebral abnormalities progressed from mild neuropathological changes such as abnormal cortical mini-columns or others.

It is believed that the frontal cerebral cortex is involved in higher cognitive functions such as undertaking of initiatives and planning of future actions in humans. The structural alteration of this cortex in Asperger's syndrome exhibits disordered social interactions. Anatomical studies and association with teratogens strongly suggest that the developmental alteration of brain occurs after conception (3). Abnormal migration of embryonic cells during fetal development changes the final structure and pathways of the brain, which results in alterations in the neural circuits that control thoughts and behaviors (4). Several theories have been proposed, but none of them can provide a complete explanation for Asperger's syndrome (5). Koechlin and Hyafil believed that a process called "cognitive branching" might be the core function of the frontal cerebral cortex (6). Cognitive branching enables a person to maintain a previously running task in a pending status for subsequent retrieval and execution upon completion of the ongoing one. Many of our mental activities and behaviors require simultaneous engagement of multiple tasks, which suggests the frontal cerebral cortex may perform a "domain-general" function in these scheduling processes. Burgess et al. proposed that there is a "supervisory attentional gateway" (SAG) system in the frontal cerebral cortex of the human brain (7). The SAG system operates under unusual conditions to ensure optimal use of cognitive resources and overcome a potential impasse that would otherwise be experienced by the system. This mechanism is involved in optimizing performance in many situations, from exploration to switching of tasks, and attention or behavioral organization over long periods of time.

In conclusion, this is the first case of cerebral abnormalities other than abnormal cortical mini-columns and gyral formations in Asperger's syndrome reported in the literature. This case expands our pathological view into Asperger's syndrome. A larger number of cases are obviously needed to investigate neuropathogenesis of this syndrome as well as other cerebral developmental disorders.

References


(Received March 23, 2012; Accepted May 11, 2012)
New opportunity for orphan drug development in Japan: Early exploratory clinical trial bases promote drug translation from basic studies to clinical application

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Summary

In Japan, although orphan drug legislation has been established in 1993 to encourage drug research and development (R&D) for intractable and rare diseases, nearly half of the orphan drugs in the Japanese market originated from the European Union (EU) or the United States of America (USA). Availability of orphan drugs for intractable and rare diseases is compounded by the "drug lag" phenomenon, which is mainly caused by the imperfect clinical trial environment in Japan. In recent years, the Japanese government paid great attention to development of innovative drugs and medical devices which originated from Japan. With financial support and institutional guarantees from government, the project of "Early Exploratory Clinical Trial Bases for Specific Research Areas" was launched in 2011 and 5 institutions were selected as the national early exploratory clinical trial bases for specific research areas including cancer, cerebral and cardiovascular diseases, neuropsychiatric disorders, and immunological intractable diseases. The early exploratory clinical trial bases offer a new opportunity for drug development for immunological and neuropsychiatric intractable diseases, thereby promoting orphan drug translation from basic studies to clinical use.

Keywords: Intractable and rare diseases, orphan drugs, clinical trial, autoimmune disease, neuropsychiatric disorders

1. Introduction

New drug development in Japan is pushed forward by the process of pre-clinical research and clinical trials. After confirmation of safety and effectiveness, pharmaceutical companies can apply to the Ministry of Health, Labour and Welfare (MHLW) for marketing approval. Currently, the time period for drug development in Japan is longer and the number of Japanese originated innovative drugs is fewer compared to that in the European Union (EU) and the United States of America (USA) (1).

Concerning drugs for intractable and rare diseases, orphan drug legislation was established in Japan in 1993 to encourage the corresponding drug research and development (R&D). The incentives mainly include financial subsidies for up to 50% of expenses for clinical and non-clinical research during the entire research process, exclusive marketing rights for 10 years (compared to 6 years for other medications), 15% tax credits on research costs excluding financial subsidies, and up to a 14% reduction in corporate tax (2,3). However, almost half of the orphan drugs in the Japanese market originated from the EU or USA although the orphan drug legislation has been implemented for nearly 20 years (3). Generally speaking, the length of drug research to market cycle is 10-12 years (4), but there is about a two-year lag period for drug approval in Japan compared with the EU and USA due to the delayed clinical trial application and the relatively longer approval period. This situation is called "drug lag" in Japan. For many drugs originated from Japan, clinical trials are first conducted in the EU or USA, though the basic research has been completed in Japan. Moreover, less drug clinical trials are led by physicians in Japan with a total number of 71 during the period between 2004 to 2010 (5).
The main cause for drug lag is that the drug clinical trial environment is less than perfect in Japan. First, inadequate cooperation between academic institutions and pharmaceutical companies makes drug R&D goals not very clear, leading to the lag from basic research to clinical trials. Second, insufficient national support measures and institutional guarantees (involving both physicians and professional clinical institutions) for being first-in-human clinical trials affect the development of drugs in clinical trials, resulting in a lag period of practical applications compared to the EU and USA. If this condition continues, the Japanese patients cannot get the most advanced drugs in a timely fashion and the international competitiveness of Japanese pharmaceutical companies will be weakened, which ultimately influences the improvement of health care levels in Japan.

2. Early exploratory clinical trial bases for specific research areas

In recent years, the Japanese government has paid great attention to the development of innovative drugs and medical devices which originated from Japan with the goals of accelerating Japanese patients getting the most advanced medical service as well as enhancing the international competitiveness of Japanese pharmaceutical companies. According to the "New Growth Strategy" and "4th Science and Technology Basic Plan" formulated by the Cabinet Office in 2010 and 2011, the "Life Innovation Project for Constructing Longevity and Healthy Society" was established. Under this project, three government departments including the MHLW, the Ministry of Education, Culture, Sports, Science and Technology (MEXT), and the Ministry of Economy, Trade and Industry (METI) jointly pushed forward development and application of innovative drugs and medical devices for specific research areas. For the specific research areas of cancer, cerebral and cardiovascular diseases, neuropsychiatric disorders, and immunological intractable diseases (Table 1). For each base, the Japanese government invests up to 500 million yen for infrastructure construction and 150 million yen for clinical trial research led by physicians (8).

3. The new opportunity for drug development for intractable and rare diseases

The confirmed 5 national early exploratory clinical trial bases include 4 bases for drugs and 1 base for medical devices. Of the 4 drug bases, 2 bases have made clear plans for development of new drugs against intractable and rare diseases. The early exploratory clinical trial base for immunological intractable diseases The School of Medicine, Keio University was confirmed as the national early exploratory clinical trial base for immunological intractable diseases. The Keio University Hospital (KUH) is one of the representative hospitals for treatment of immunological intractable diseases. Established in 2010, Keio University Hospital Immunology Integrated Medical Care Center has conducted several studies concerning diagnosis and treatment of immunological intractable diseases such as Crohn's disease, ulcerative colitis, systemic lupus erythematosus, and rheumatoid arthritis (9,10). Since it was selected as a national early exploratory clinical trial base in 2011, KUH has strengthened construction of infrastructure facilities with support of governmental finance and policy under the mission of R&D of novel drugs for patients with immunological intractable diseases. Meanwhile, the base

Table 1. National early exploratory clinical trial bases for specific research areas in Japan

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<thead>
<tr>
<th>Institution</th>
<th>Type</th>
<th>Research Area</th>
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<tr>
<td>National Cancer Center Hospital East</td>
<td>Drug</td>
<td>Cancer</td>
</tr>
<tr>
<td>Osaka University Hospital</td>
<td>Drug</td>
<td>Cerebral and cardiovascular disease</td>
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<tr>
<td>National Cerebral and Cardiovascular Center</td>
<td>Medical device</td>
<td>Cerebral and cardiovascular disease</td>
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<tr>
<td>The University of Tokyo Hospital</td>
<td>Drug</td>
<td>Neuropsychiatric disorder</td>
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<tr>
<td>School of Medicine, Keio University</td>
<td>Drug</td>
<td>Immunological intractable disease</td>
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cooperates with other departments of Keio University such as the pharmaceutical supervision lab and clinical drug evaluation lab in the School of Pharmaceutics in order to construct a comprehensive system containing drug safety, efficacy, and supervision evaluation.

The early exploratory clinical trial base for neuropsychiatric intractable diseases The University of Tokyo Hospital (UTH) was confirmed as the national early exploratory clinical trial base for neuropsychiatric disorders. One of the major tasks is to promote development of drugs for Alzheimer’s disease (AD), which is one of the neuropsychiatric intractable diseases. With society aging, the number of patients afflicted by AD has increased. However, there are no drugs for curing this disease thus far. This situation is compounded by lack of quantifiable methods for evaluation of drug efficacy. Since 2008, in working towards a disease-modifying therapy for AD, The University of Tokyo has carried out studies for the Japanese AD Neuroimaging Initiative (J-ADNI) and explored quantification for evaluation of AD drugs by using brain imaging technology such as positron emission tomography-computed tomography (PET-CT) (11,12). Considering the rate of disease progression and the invisibility of drug efficacy, it is necessary to conduct the trials in patients in the early phase of AD. Applying the J-ADNI technology, doctors in UTH managed clinical trials to confirm the rate of disease progression and the efficacy of drug treatment. Since being confirmed as a national early exploratory clinical trial base in 2011, the base is dedicated to construct infrastructure facilities including human resources and equipments with support of governmental finance and policy. The first-in-human clinical trials in healthy volunteers is programmed to be conducted in 2013 and clinical trials in patients in the early phase of AD or mild cognitive impairment will be carried out in 2014 after confirmation of drug safety and clarification of pharmacodynamics.

4. Conclusion

A drug lag phenomenon exists in drug R&D in Japan. The main cause of this phenomenon is that the environment for drug clinical trial bases is not perfect, which leads to delay of clinical studies compared with the EU and USA. In 2011, the project of “Early Exploratory Clinical Trial Bases for Specific Research Areas” was launched with the support of the MHLW, MEXT, and METI. Five health institutions were selected as the national early exploratory clinical trial base for specific research areas, which provides opportunities for orphan drug R&D. With the support of governmental finance and policy, novel drugs targeting immunological and neuropsychiatric intractable diseases will enter into early exploratory clinical trials at the bases of KUH and UTH. Cooperation among government, basic research institutions and pharmaceutical companies will promote the process of drug translation from basic studies to clinical application, accelerate the availability of drugs for intractable and rare diseases in Japan, and enhance the international competitiveness of Japan original medicines, thereby increasing the medical level of intractable and rare diseases treatment in Japan.

Acknowledgement

This work was supported by the International Research and Cooperation Association for Bio & Socio-Sciences Advancement Group for Rare Diseases Research.

References


(Received March 1, 2012; Accepted March 9, 2010)
Guide for Authors

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Original Articles should be well-documented, novel, and significant to the field as a whole. An Original Article should be arranged into the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References. Original articles should not exceed 5,000 words in length (excluding references) and should be limited to a maximum of 50 references. Articles may contain a maximum of 10 figures and/or tables.

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