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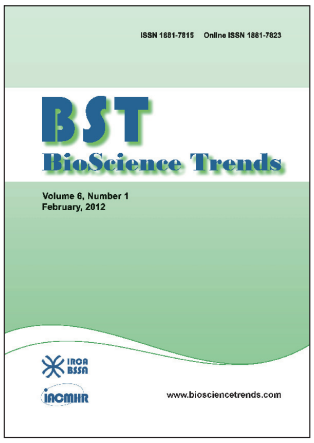

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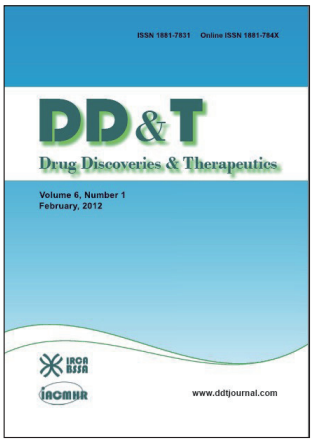

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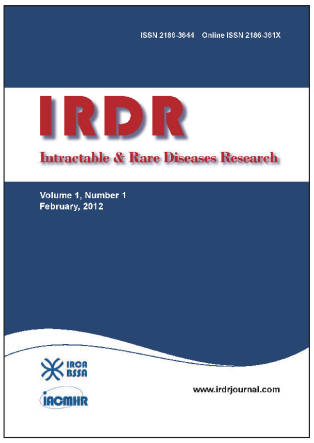

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Editorial and Head Office

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

Tel: +81-3-5840-9968, Fax: +81-3-5840-9969
E-mail: office@irdrjournal.com
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Alzheimer's Disease and Prion Protein

Jiayi Zhou[1,*], Bingqian Liu[2]

[1] *Department of Biochemistry, Boston University School of Medicine, Boston, MA, USA;*
[2] *State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, Guangdong, China.*

Summary **Alzheimer's disease (AD) is a devastating neurodegenerative disease with progressive loss of memory and cognitive function, pathologically hallmarked by aggregates of the amyloid-beta (Aβ) peptide and hyperphosphorylated tau in the brain. Aggregation of Aβ under the form of amyloid fibrils has long been considered central to the pathogenesis of AD. However, recent evidence has indicated that soluble Aβ oligomers, rather than insoluble fibrils, are the main neurotoxic species in AD. The cellular prion protein (PrP^C) has newly been identified as a cell surface receptor for Aβ oligomers. PrP^C is a cell surface glycoprotein that plays a key role in the propagation of prions, proteinaceous infectious agents that replicate by imposing their abnormal conformation to PrP^C molecules. In AD, PrP^C acts to transduce the neurotoxic signals arising from Aβ oligomers, leading to synaptic failure and cognitive impairment. Interestingly, accumulating evidence has also shown that aggregated Aβ or tau possesses prion-like activity, a property that would allow them to spread throughout the brain. In this article, we review recent findings regarding the function of PrP^C and its role in AD, and discuss potential therapeutic implications of PrP^C-based approaches in the treatment of AD.**

Keywords: *PRNP* gene, protein misfolding, N1 fragment, Fyn kinase, long-term potentiation (LTP)

1. Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases, characterized clinically by progressive loss of memory and decline in cognitive function and pathologically by cerebral accumulation of amyloid-beta (Aβ) peptides in extracellular senile plaques and formation of intracellular neurofibrillary tangles constituted by hyperphosphorylated tau protein. The two pathological events are thought to be sequentially associated (*1,2*). AD is named after the German psychiatrist and neuropathologist Alois Alzheimer who first described the disease in 1907 (*3-5*). It is the most common form of dementia, while being a leading cause of death or disability. AD occurs most often in people over 65 years of age, although a less-prevalent early-onset type can occur much earlier. Around 35 million people are estimated to be afflicted with AD worldwide, and the incidence rises exponentially with advancing age, posing a huge challenge for society and health care (*6*). There are no treatments so far to cure, delay or stop the disease progression. Although the etiology of AD is not fully understood, accumulation of amyloid-beta (Aβ) peptides in the brain is considered the causative component of AD pathogenesis (amyloid hypothesis) (*7-10*). Aβ peptides of varying length are produced by sequential cleavage of amyloid precursor protein (APP) by β-secretase (mostly β-site APP-cleaving enzyme 1, BACE1) and gamma-secretase (*11-14*). Compared to Aβ 40 (peptide with 40 amino acid residues), the longer form Aβ 42 (peptide with 42 amino acid residues) has an increased propensity to oligomerize and aggregate to form fibrillar amyloid plaques in the brain, and is widely regarded as the main pathogenic species causing AD. Genetic mutations in APP or preselinin-1 (PS-1) or PS-2 (catalytic subunits of gamma-secretase) lead to overproduction of Aβ 42, and cause early onset AD (*15*).

Upon failure of all the Aβ-centric approaches that reached Phase III clinical trials, scientists began to question the pathogenic role of amyloid aggregates (senile plaques) that comprise Aβ fibrils, which is the main theme of the amyloid hypothesis (*10*), and speculate that

**Address correspondence to:*
Dr. Jiayi Zhou, Department of Biochemistry, Boston University School of Medicine, 72 East Concord Street, Boston, MA 02118, USA.
E-mail: jzhou10@bu.edu

the soluble pre-fibrillar Aβ oligomers are most likely the principal toxic forms of Aβ peptide (*16-20*). Soluble Aβ oligomers are found to be elevated in AD brains, and their levels are strongly correlated with disease onset and severity (*21-23*). The cellular mechanisms of Aβ oligomer-mediated neurotoxicity are poorly understood. Recent evidence indicate that the Aβ oligomers, also referred to as amyloid-derived diffusible ligands (ADDLs) (*22,24,25*), may bind to a surface receptor on neurons, thereby initiating signaling transduction pathways that lead to synaptic dysfunction and neuronal death (*26-29*). One interesting receptor for Aβ oligomers so far identified is the cellular prion protein (PrP^C) (*30*), which is a cell membrane glycoprotein ubiquitously expressed but enriched in the brain.

The discovery of PrP^C as a cell surface receptor for Aβ oligomers has sparked a major interest in research focusing on identification of downstream effectors that mediate the neuronal toxicity and synaptic dysfunction in AD. This subject will be discussed in details later.

2. Cellular prion protein (PrP^C)

Prion diseases are a group of fatal infectious neurodegenerative diseases comprising Creutzfeldt-Jacob disease (CJD), variant Creutzfeldt-Jacob disease (vCJD), Gerstmann-Straussler-Scheinker disease (GSS), fatal familial insomnia (FFI), and Kuru in humans, as well as bovine spongiform encephalopathy (BSE) (otherwise known as mad cow disease) and scrapie in animals (*31,32*). In prion diseases, the normal PrP^C is converted into the β-sheet rich, protease-resistant pathogenic form – scrapie prion protein (PrP^{Sc}), which is infectious and spreads throughout the brain (*33*). Stanley Prusiner at the University of California San Francisco (UCSF), USA, first described in 1982 that novel proteinaceous infectious particles (prions) could replicate and propagate without nucleic acids and cause scrapie (*32*).

PrP^C is encoded by the *PRNP* gene (PRioN Protein) on chromosome 20 in human and the corresponding chromosome 2 in mouse (*34,35*). PrP^C is synthesized in the endoplasmic reticulum (ER) and transits the Golgi on its way to the cell surface. The structure of mouse PrP^C is illustrated in Figure 1 (*36*). Post-translational modifications of PrP^C include removal of the N-terminal signal peptide (residues 1-22), N-linked glycosylation at Asn-180 and Asn-196, formation of a disulfide bond between residues 178 and 231, and attachment of a glycosylphosphatidylinositol (GPI) anchor following removal of the C-terminal hydrophobic peptide (residues 231-254) (*36-39*), rendering a mature PrP^C with about 210 amino acid residues and a molecular weight of 33-35 kDa. PrP^C is almost ubiquitously expressed across tissues, with an enrichment in synaptic membranes and astrocytes in the brain.

The precise physiological function of PrP^C is still unknown. Several pieces of evidence have shown that PrP^C plays a role in metal ion trafficking (*40,41*), cell adhesion (*42-44*), cell survival (*36,45*), immune regulation (*46,47*) and signal transduction (*26,48,49*). *PRNP* gene knockout mice are developmentally normal and have no signs of neurodegeneration (*50,51*), indicating that the prion pathology is unlikely to be the result of a loss of PrP^C function. On the contrary, depletion of neuronal PrP^C is protective and reverses the disease pathology in scrapie-infected mice (*52*), presumably due to depletion of the substrate for generation of PrP^{Sc} (*53*). Scrapie infection of transgenic mice expressing PrP^C lacking the GPI anchor causes efficient prion replication, but no pathology (*54*). Therefore, normal GPI-anchored PrP^C is required for the neurotoxicity of PrP^{Sc} (*55,56*).

3. PrP^C: a receptor to mediate Aβ toxicity

It is now widely accepted that the soluble Aβ oligomers are the toxic species that leads to synaptic and cognitive dysfunction as well as neurodegeneration in AD (*17,25*). This concept is supported by studies showing the strong correlation of the synaptic loss with cortical levels of soluble Aβ species rather than with plaque distribution in AD patients (*20,21,24*), and the inhibitory effect of soluble Aβ oligomers on long-term potentiation (LTP)

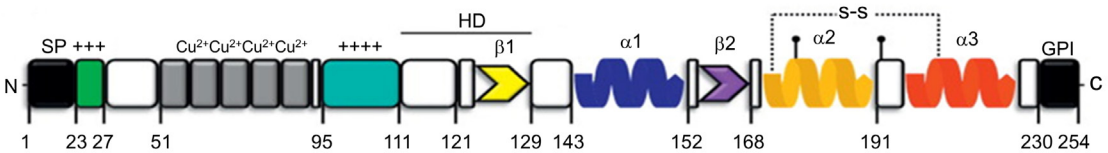

Figure 1. Scheme of PrP^C primary structure. The N-terminal part includes (from left to right): a signal peptide (SP, residues 1-22) (removed during PrP biosynthesis in the endoplasmic reticulum), a polybasic region (residues 23-27, green), five histidine-containing octapeptide repeats (residues 51-90, gray) (bind Cu^{2+} and other bivalent metal ions), a central region (CR) (residues 95-111, cyan, positively charged), and a hydrophobic domain (HD, residues 111-130, highly conserved region). The C-terminal part includes (from left to right): two short β-strands (residues 127-129, yellow; and 166-168, purple), three α-helices (residues 143-152, blue; 171-191, orange; and 199-221, red), and a C-terminal peptide (residues 231-254, black), which is removed during biosynthesis, followed by covalent attachment of a glycosylphosphatidylinositol (GPI) anchor, which attaches the protein to the outer leaflet of the plasma membrane. PrP^C also contains two N-linked oligosaccharide chains (at Asn-180 and Asn-196, black lollipops) and a disulfide bond between residues 178 and 231 (indicated by a dashed line). Residues correspond to the mouse sequence. This figure is adapted from Biasini *et al*. Trends in Neurosciences (2012) with permission.

(*19,22,57-59*).

The mechanism of Aβ oligomer toxicity remained largely unknown until the Strittmatter's group at Yale University identified PrP^C as a receptor capable of mediating the neurotoxic effect of Aβ oligomers (*30*). To identify candidates for this receptor, they conducted an unbiased expression-cloning screen of a brain cDNA library for binding sites with an oligomeric preparation of synthetic biotinylated Aβ 42 peptides. From more than 225,000 expressed clones, two independent positive clones were isolated and both were found to encode full-length mouse PrP. They found that Aβ 42 oligomers bound to PrP with high affinity and specificity. They then tested the function of this interaction in cultured hippocampal slices, and found that nanomolar concentration of Aβ 42 oligomers potently suppressed CA1 hippocampal LTP and this suppression was not observed in slices generically lacking PrP^C or in presence of an anti-PrP antibody that blocks the binding of Aβ 42 oligomers to PrP^C, indicating that the suppression of LTP is specifically mediated by binding of Aβ 42 oligomers to PrP^C (*30*). Therefore, they have provided compelling evidence that PrP is a specific binding partner for Aβ 42 oligomers and mediates the inhibitory effect of Aβ 42 on synaptic plasticity.

The Strittmatter's group then sought to test *in vivo* whether PrP^C is essential for the ability of brain-derived Aβ to suppress cognitive function. They crossed familial AD transgenes encoding APPswe and PSen1DeltaE9 into *Prnp*$^{-/-}$ mice, and found that mice lacking PrP^C, but containing Aβ plaques derived from APPswe/PSen1DeltaE9 transgenes, showed no detectable impairment of spatial learning and memory, while the AD transgenic mice with intact PrP^C exhibited dramatic deficits in spatial learning and memory, indicating that PrP^C is selectively required for the toxicity of the naturally occurring Aβ in the brain that leads to the cognitive phenotypes in these AD transgenic mice (*60*), which is in consistency with previous reports that Aβ oligomers isolated from the brain of Alzheimer's patients (*20,23,59,61*) requires PrP^C to suppress LTP (*62,63*).

The Strittmatter's group further found that soluble Aβ assemblies derived from the brains of individuals with Alzheimer's disease interacted with PrP^C at the postsynaptic density to activate the Src kinase Fyn, which phosphorylates the NR2B subunit of NMDA receptor and causes transient increase of NR2B on the cell surface with consequent excitotoxicity, while rendering destabilization of dendritic spines. Both NR2B phosphorylation and spine destabilization incurred by Aβ oligomers were eliminated in *Prnp*$^{-/-}$ and *Fyn*$^{-/-}$ neurons, indicating a specific association of Aβ-PrP^C-Fyn-mediated toxic signaling (*26,64*). This study sheds new light on the molecular mechanism of PrP^C-mediated Aβ toxicity, while indicating a **prion** connection of Aβ and Fyn (*22,49*). Another group further demonstrated that soluble Aβ binds to PrP^C at neuronal dendritic spines, where it forms a complex with Fyn, and results in the activation of the kinase and subsequent Fyn-dependent tau hyperphosphorylation in a *PRNP* gene dose-dependent manner (*2*), making another **prion** connection that links together the two hallmark pathological events in AD – amyloid accumulation and tau hyperphosphorylation (*22,65,66*). However, how binding of Aβ oligomers to PrP^C activates Fyn still remains enigmatic. PrP^C is an extracellular protein attached to the outer surface of the cell membrane by a GPI anchor, and Fyn is located in the cytoplasm. Current evidence indicates that factors like caveolin-1 or the neural cell adhesion molecule (NCAM) could potentially connect PrP^C and Fyn from the two opposite sides of the cell membrane (*48,67-69*).

Two distinct binding sites for Aβ oligomers have been identified on PrP^C by deletion analysis, antibody binding (*30*), and biophysical techniques such as site-directed spin labeling and surface plasmon resonance (*70*). Both sites are rich in positively charged basic residues: one is immediately adjacent to the central region (residues 95-110) and the other is at the extreme N-terminus (residues 23-27) (Refer to Figure 1). It is very likely that the two sites act in concert to render high affinity binding for Aβ oligomers, and deletion of either region results in a major loss of the binding capacity (*36,70*).

4. PrP^C: a tale of an "evil angel"

As we know, PrP^C is converted into an aggregated, β-sheet-rich neurotoxic isoform called PrP^{Sc} in prion diseases (*33,71*). PrP^C serves not only as the substrate for PrP^{Sc} conversion and propagation, but also as a transducer of PrP^{Sc}-associated neuronal death (*52,56,72*). Another noxious function of PrP^C is to serve as a cell surface receptor for Aβ oligomers to mediate signal transduction leading to neuronal toxicity (*26,30,60*), which we have already discussed above.

However, this "evil" protein has been found to have numerous beneficial "angel" functions. One notable function is that PrP^C suppresses glutamate-mediated neuronal excitatoxicity by inhibiting NMDA receptor (*36,73*). Another function is that PrP^C physically interacts with the APP cleaving enzyme BACE1 through its N-terminal polybasic domain (residues 23-26) and inhibits its enzyme activity, resulting in a reduction of Aβ production (*74-76*), which indicates a preventive role against AD. In both cell and animal models, PrP^C has been shown to lower Aβ production by inhibiting BACE1 (*75,76*).This function is thought to be modulated by *PRNP* polymorphism at codon 129 (M129V), which may be associated with increased risk of AD (*77-81*). Interestingly, binding of Aβ oligomers to PrP^C impairs the inhibitory effect of PrP^C on BACE1 activity (*64*), which may indicate another mechanism of Aβ oligomer toxicity.

A physiological process that makes PrP^C a "double-

faced gem" is that PrP^C undergoes constitutive proteolytic cleavage between residues 111/112, yielding a soluble N-terminal fragment (N1) and a membrane-bound C-terminal fragment (C1), which have a protective role in AD and prion disease, respectively. N1 binds to Aβ oligomers with high affinity, and blocks the neurotoxicity of Aβ oligomers through neutralizing toxic assemblies of Aβ. Therefore, N1 may serve as a potent inhibitor of Aβ oligomer toxicity and represent an entirely new class of therapeutic agents for the treatment of AD (*82*). N1 is a naturally occurring soluble fragment that is generated by endogenous proteolytic processing of PrP^C at the α-site (residues 111 and 112) (*83*), presumably by ADAM (a disintegrin and metalloprotease) proteases (*84-87*). Blocking ADAM10 synaptic trafficking has been shown to be able to generate a model of sporadic Alzheimer's disease (*88*). Agents that could stimulate α-cleavage of PrP^C should be good drug candidates. On the other hand, the C1 fragment of PrP^C can inhibit PrP^{Sc} formation and accumulation of neurotoxic forms of PrP. The C1 transgenic mice inoculated with PrP^{Sc} were found healthy and did not exhibit PrP^{Sc} accumulation, indicating that C1 is not a substrate for conversion to PrP^{Sc}. Manipulating C1 fragment may thus have therapeutic value for prion diseases (*89*).

PrP^C homodimerization has been found to be an important regulator of PrP^C α-cleavage and stimulate the production of N1 and C1 fragments. The increase of N1 is protective against the toxicity of Aβ oligomers. Thus, manipulation of PrP^C homodimerization may represent a potential therapeutic avenue against Aβ toxicity in Alzheimer's disease (*90*). Interestingly, the APP processing enzyme α-secretase (belongs to ADAM family of zinc metalloproteases), which precludes Aβ production by cleaving APP within the Aβ domain (*91*), also cleaves PrP^C at the α-site (residues 111 and 112), releasing N1 from the membrane. Therefore, enhancing the activity of α-secretase may represent a novel therapeutic strategy by reducing the toxic Aβ production and increasing the protective N1 production (*92*).

5. AD: a story of two prions

The misfolding and aggregation of Aβ and tau proteins were traditionally thought to contribute in parallel to pathogenesis of AD. Accumulating evidence indicated that misfolded, toxic oligomers of Aβ and tau spread through the brain in a way much like misfolded PrP (*93-95*). The misfolded forms of Aβ or tau have a seeding effect, and can induce normal Aβ or tau in the cells to misfold, spread and become toxic (*96-102*). Therefore, AD can be regarded as a disease that harbors two proteins with prion-like behavior: Aβ and tau (*103,104*). The prion-like propagation of additional proteins whose misfolding into β-sheet-rich structures underlies other well-known neurodegenerative diseases has also been indicated (*105-107*). Thus, a prion-based mechanism is proposed to unite a wide array of neurodegenerative diseases, all of which may stem from misfolded proteins self-propagating through the brain (*103,108*). Local injection of misfolded Aβ in the brains of AD transgenic mice has been found to trigger the misfolding and spreading of otherwise normal Aβ throughout the brain, indicating the prion-like activity of Aβ (*108-114*). Injection of AD brain extracts into the hippocampus of mice expressing human wild-type APP induces Aβ deposition, which progressively increases over time after inoculation and spreads to brain areas far from the injection site, where other Aβ-related pathology is also observed (*114*). It is believed that certain Aβ conformations tend to self-propagate, and targeting those specific Aβ assemblies may interrupt the spread of Aβ deposition, hence exerting therapeutic effect on AD (*109*).

Intracerebral injection of aggregation-prone mutant tau in mice has also been demonstrated to induce wild-type tau to form neurofibrillary tangles and spread throughout the brain (*99*). Accumulating evidence indicates that Aβ works upstream of tau in AD pathogenesis (*65,115-121*). Aβ can bind to tau and induce formation of tau oligomers, which can then self-propagate without additional Aβ, indicating a cross talk between the two prions (*104*).

6. PrP^C: a novel therapeutic target for AD

PrP^C has been identified as a major player in mediating the toxicity of Aβ oligomers that leads to synaptic loss and cognitive impairment in AD. Therefore, targeting PrP^C, its interaction with Aβ oligomers, or downstream mediators can be considered the new line of choice for therapeutic development for treatment of Alzheimer's disease.

Genetic ablation of PrP^C in mice rescues the neurotoxic phenotypes of Aβ oligomers (*30,60*). It might be reasonable to speculate that using shRNA or siRNA to knock down the expression of PrP^C may represent a therapeutic approach for AD, though little has been done in this regard. Nevertheless, knocking down PrP^C will also affect other functions of PrP^C, causing various complications. For example, PrP^C reduces Aβ production by inhibiting BACE1 activity, and has a protective role in AD (*74,75*). This can be jeopardized by PrP^C knockdown.

Attempts have been made to seek antibodies that could efficiently bind to PrP^C and block the binding site(s) of Aβ oligomers, which may have therapeutic effect on AD by preventing the Aβ oligomer/PrP^C-initiated noxious signaling. Michael Rowan group at University College Dublin in the U.K. found that antibodies against the epitopes at the PrP principal Aβ-binding site and helix-1 were able to block Aβ binding and block the Aβ-mediated disruption of synaptic

plasticity (*63*). Chung *et al*. from New York University School of Medicine intraperitoneally injected the monoclonal anti-PrP antibody, 6D11, in APP/PS1 transgenic mice, and found that the treatment with 6D11 antibody completely rescue the cognitive and behavioral deficits of the transgenic animals (*122*). The 6D11 antibody is directed against the epitope (residues 93-109), which is the region suggested to be involved in Aβ oligomer binding.

Screening for small molecules that could efficiently target either the Aβ oligomer/PrP^C interaction or the downstream mediators may represent a promising avenue for therapeutic development.

The Fyn kinase has been found to be activated upon binding of Aβ oligomers with PrP^C, which then initiate downstream signaling to mediate Aβ toxicity, for example, activation of Fyn kinase lead to hyperphosphorylation of tau (*2,66*). Targeting Fyn kinase or other Aβ/PrP^C downstream mediators, for example by genetic engineering, RNAi, or small molecule modulators, may also be of therapeutic value.

Synthetic N1 fragment, equivalent of that released by α-cleavage of endogenous PrP^C, has been found to bind Aβ oligomers with high affinity, sequester Aβ oligomers in the extracellular space, and hence block the Aβ oligomer-mediated synaptic toxicity (*82*). Therefore, exogenous administration of N1 or enhancement of endogenous α-cleavage of PrP^C represents a brand-new class of therapeutic approaches for AD. Among others, seeking modulators that prevent Aβ oligomerization or inhibit the prion-like activity of Aβ or tau may represent another category of therapeutic development strategies for the treatment of AD.

The 'PrP^C axis' of therapeutic development strategies for AD is illustrated in Figure 2.

7. Discrepancies

The Strittmatter group's discovery of PrP^C as an Aβ oligomer receptor to mediate synaptotoxicity has created an exciting hot spot, which has greatly stimulated research in the field. However, studies from different groups around the world showed discrepancies and some groups came to a completely opposite conclusion to the Strittmatter group's, which made scientists begin to question the role of PrP^C in mediating the toxic effects of Aβ oligomeric assemblies.

The work published in Nature a year later from Roberto Malinow's group at the University of California at San Diego in the USA reported that PrP^C is not required for Aβ-induced synaptic toxicity, having raised a conflicting concern that Aβ-mediated synaptic defects do not require PrP^C. The Aβ-induced depression of synaptic transmission was observed in both wild-type and *Prnp*$^{-/-}$ mouse slices (*123*).

Gianluigi Forloni and his team at the Mario Negri Institute for Pharmacological Research in Italy injected Aβ 42 oligomers into the lateral ventricle of C56BL/6 mice and found that PrP-expressing and PrP knock-out mice were equally susceptible to cognitive impairment,

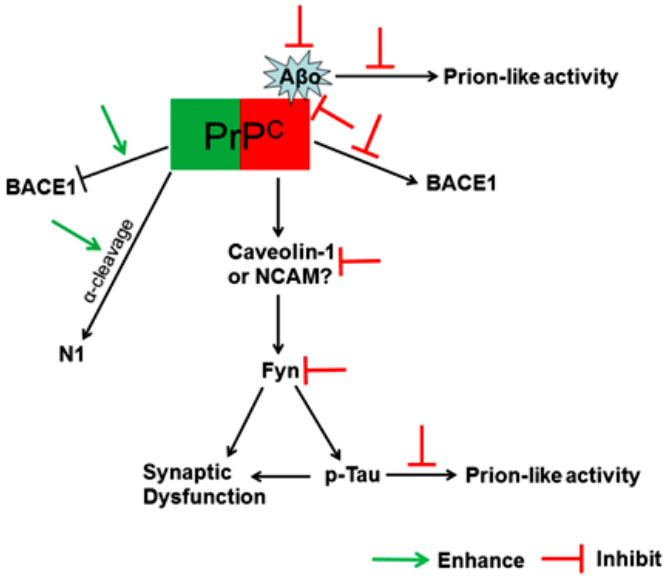

Figure 2. Therapeutic development strategies for AD (The 'PrP^C axis'). The green color portion of PrP^C indicates the protective function of PrP^C (left-hand side), and the red color portion of PrP^C indicates PrP^C as the receptor of Aβ oligomers (right-hand side) to mediate the Aβ toxicity. The left-hand side of the figure indicates therapeutic strategies by enhancing the normal function of PrP^C (enhancing the inhibitory effect on BACE1, which reduces Aβ production, and enhancing the α-cleavage, which increases production of the protective N1 fragment of PrP^C). The right-hand side of the figure indicates therapeutic strategies by targeting the Aβ oligomer/PrP^C-mediated toxic signaling pathway, which encompass measures to inhibit Aβ oligomerization, Aβ prion-like activity, interaction of Aβ oligomers with PrP^C, Aβ oligomer/PrP^C-mediated disinhibition of BACE1, intermediate mediators (such as caveolin-1 and NCAM), Fyn kinase, and prion-like activity of hyperphosphorylated Tau. (Abbreviations: Aβo: Aβ oligomers; N1: N1 fragment of PrP^C; p-Tau: hyperphosphorylated Tau.)

suggesting that PrP^C is not required for Aβ 42 oligomer-mediated cognitive impairment, although they in the meantime confirmed that Aβ 42 oligomers do interact with PrP^C with nanomolar affinity (*124*).

Andriano Aguzzi and his group at the University Hospital Zurich in Switzerland have also reported findings that challenge the role of PrP^C as an Aβ toxicity mediator. They found that deletion or overexpression of PrP^C had no effect on the impairment of hippocampal synaptic plasticity, while having also confirmed the efficient binding of Aβ 42 oligomers to PrP^C (*125*), once again showing contradictory results to those of Strittmatter and his team (*30,60*).

However, those conflicting reports do not necessarily negate the findings of Strittmatter and his team. They might arise from differences in animal models, experimental settings, and preparations of Aβ oligomers (method of preparation, material source, and size and conformation of Aβ oligomeric assemblies may all matter) (*126*).

A recent study from Michael Rowan group at University College Dublin in the U.K., which we have mentioned previously, clearly shows that PrP is required for the plasticity-impairing effects of toxic Aβ species from human AD brain and that standardized ADDL preparations disrupt hippocampal synaptic plasticity in a PrP-dependent manner (*63*). They further found that antibodies that block Aβ binding to PrP^C block the toxic effect on synaptic plasticity.

Sylvain Lesne group at the University of Minnesota in the USA has recently demonstrated that soluble Aβ binds to PrP^C at neuronal dendritic spines, where PrP^C is enriched, and causes hyperphosphorylation of tau by activation of the Fyn kinase. The PrP^C antibody 6D11 prevents Aβ oligomers from binding to PrP^C, and abolishes subsequent Fyn activation and Fyn-dependent tau hyperphosphorylation.

There are also other studies that have indicated a role of PrP^C in mediating the toxicity of Aβ oligomers, supporting the findings of the Strittmatter group (*64,127*). Therefore, the argument is far from conclusive.

8. Concluding remarks and perspectives

Although there are conflicting reports regarding the function of PrP^C as a cell surface receptor to mediate the deleterious effects of Aβ oligomers in AD, there are no ambiguities for two end points: high affinity binding of Aβ oligomers to PrP^C (*30,70,124,125*), and high synaptic toxicity of Aβ oligomers (*23,59-61*). The challenge remaining for scientists is to 'make the two ends meet'.

Strittmatter group's finding that PrP^C acts as the receptor for mediating Aβ oligomer neurotoxicity (*30*) has at least opened a new direction towards understanding the molecular mechanism that connect Aβ oligomers and their toxic effects. The research discoveries of his team have apparently been supported by multiple studies from other groups, though there have been conflicting results reported. Sophisticated studies using advanced animal models and optimized experimental conditions are needed to elucidate the precise role of PrP^C in mediating Aβ oligomer neurotoxicity, or to identify other potential cell surface receptors and signaling networks that make the two ends – toxin and toxicity – meet.

If the role of PrP^C in Aβ oligomer-mediated pathogenic process turns out to be substantial, it would be of interest to seek potential co-receptors or to examine whether other Aβ-mediated signaling pathways are also PrP^C-dependent, making a complete **prion** connection network of AD.

To conclude, endeavors to gain precise understanding of the Aβ oligomer-mediated neurotoxic signaling pathways will greatly facilitate the development of novel therapies that would be able to target specific Aβ oligomeric assemblies and their downstream associates, and offer new hope to AD patients and families. Identification of the biophysical features of naturally occurring toxic Aβ species in human AD brain would be of critical reference to clinically relevant translational research.

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Review

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Advances in research on and diagnosis and treatment of achondroplasia in China

Yao Wang[1], Zeying Liu[2], Zhenxing Liu[2], Heng Zhao[2], Xiaoyan Zhou[2], Yazhou Cui[2], Jinxiang Han[2,*]

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

Summary **Achondroplasia is a rare autosomal dominant genetic disease. Research on achondroplasia in China, however, has received little emphasis. Around 80-90% of cases of neonatal achondroplasia result from mutations in fibroblast growth factor receptor 3 (*FGFR3*) according to polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). Recently, genetic research on achondroplasia in China made a major breakthrough by revealing two novel mutations located on the *FGFR3* gene, thus helping to complete the pathological molecular map of achondroplasia. There are still, however, unknown aspects of the diagnosis and treatment of achondroplasia. This review will summarize advances in research on and the clinical diagnosis and treatment of achondroplasia in China.**

Keywords: Rare diseases, gene mutation, chondrodystrophia fetalis, chondrodystrophic dwarfism

1. Introduction

Achondroplasia (MIM: 100800) is an autosomal dominant genetic disease that is also known as chondrodystrophia fetalis or chondrodystrophic dwarfism. Features of achondroplasia are distinctively identifiable. Patients have a large head with frontal bossing, midface hypoplasia, short limbs, trident hands, and muscular hypotonia. Commonly, these patients have recurrent ear infections, delayed motor milestones, and eventually develop bowed legs; luckily, patients with achondroplasia generally have normal intelligence (*1*). Ain *et al.* (*2*) found that 95% of six-month-old newborns with achondroplasia have the deformities mentioned earlier, and these defects progress with age. The body size of adult patients with achondroplasia is relatively small, with an average height of 131 cm for men and 124 cm for women and an average weight of 55 kg for men and 46 kg for women (*3*). X-rays have clearly shown that achondroplastic patients exhibit developmental disorders including an underdeveloped skull base/facial bones, small facial bones, an enlarged head, a prominent forehead, bullet-shaped vertebral bodies, shorter anteroposterior diameter of the spinal canals, thicker long bones as well as metaphyseal flaring (*4*,*5*). Additionally, a third of patients with achondroplasia may develop spinal stenosis and thoracolumbar kyphosis (*6*,*7*). Leg and lower back pain are reported in half of adult patients, revealing the first signs of spinal stenosis. These symptoms may appear early and can be mediated by treatment with anti-inflammatory drugs, such as periradicular corticosteroid injections for lumbar radiculopathy. A number of associated factors are considered to play an aggravating role and have to be minimized through adequate physiotherapy to prevent lumbar lordosis and/or prevention of excess weight.

Achondroplasia is a rare disease worldwide but it has a 100% rate of expression. According to statistics from western research institutes, achondroplasia has a global incidence about 1/77,000-1/15,000 (*8*). 80-90% of newborns with this condition have a sporadic case caused by mutation, and 10-20% of newborns with this condition have a familial/genetic form. Fibroblast growth factor receptor 3 (*FGFR3*) is currently known to be the only gene that causes achondroplasia, mutations in this gene lead to an abnormal protein (*9-13*).

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

The understanding of and research on achondroplasia started later in China than in Europe and the United States. From the 1980s to 1990s, nearly 60 clinical cases were reported around the country (*14*). Since then, there have been no exact statistical data on the incidence of achondroplasia in China. As a result, there is a low level of medical evidence and a lack of experience diagnosing this disease. Therefore, this review aims to summarize advances in research on and clinical diagnosis and treatment of achondroplasia in China.

2. Genetic aspects of achondroplasia

2.1. *Mutations of FGFR3 are associated with achondroplasia*

Achondroplasia is a rare autosomal dominant disorder. *FGFR3* is currently known to be the only gene that causes achondroplasia. FGFR3 is one of the key FGF binding tyrosine kinase receptors and is highly conserved in both humans and mice. The human *FGFR3* gene is located on chromosome 4q16.3 (*15*). Research has shown that FGFR3 is expressed in different tissues including cartilage, the brain, kidneys, and the intestine in different stages of development (*16*). FGFR3 is a single-pass transmembrane receptor and is involved in regulating cartilage and varied aspects of long bone development, including chondrocyte proliferation and cartilage matrix calcification. The *FGFR3* gene is 15 Kb and contains 19 exons and 18 introns. Numerous functional domains are encoded by the *FGFR3* gene, including an extracellular glycosylation ligand-binding domain, a hydrophobic transmembrane domain, and an intracellular tyrosine kinase catalytic domain (*17*). In 1994, Shiang *et al*. (*18*) first reported a mutation of *FGFR3* in the hydrophobic transmembrane domain in patients with achondroplasia according to polymerase chain reaction (PCR) combined with single-strand conformation polymorphism (SSCP). The study by Shiang *et al*. indicated that the hydrophobic transmembrane domain may be the key genetic hot zone essential to regulating cartilage development. A study by Perez-Custro *et al*. (*19*) confirmed the location of the mutation at exon 10, which encodes the hydrophobic transmembrane domain.

Genetically, around 99% of achondroplasia cases are caused by the c.1138G→A and c.1138G→C mutations. Both mutations convert glycine (Gly) into arginine (Arg) on the 380th amino acid, leading to dysfunctional proteins (*20*). In 1995, Swedish and Japanese research groups found a third base mutation – c.1123G→T – in individual cases and one family, but the incidence of this mutation is very low (about 1-2% of all mutations) (*21,22*). Recently, Prinos *et al*. reported another novel mutation, Gly to Glu on the 346th amino acid (*23*) (Table 1).

FGFR3 mutations generate deficient proteins that affect chondrocyte proliferation and calcification and hinder cartilage growth and development. In *FGFR3* knock-out mice, cartilage and long bones grow but the growth of other bones is delayed, indicating that FGFR3 inhibits bone growth by limiting chondrocyte proliferation and that it acts as a negative regulator of bone growth (*24*). In brief, *FGFR3* mutations reduce chondrocyte proliferation and limit the growth of cartilage and long bones, thereby resulting in an external phenotype of achondroplasia.

Aberrant downstream signaling of ligand-receptor interaction of FGF3 and FGFR3 is also another key factor affecting achondroplasia (*25*). Binding of FGF ligands to FGFR3 leads to activation and dimerization of the receptor and can sequentially activate target tyrosine kinase of FGFR3, leading to autophosphorylation of selected tyrosine residues in the cytoplasmic domain of the receptor (*26*). A recent study showed that FGFR3 signaling inhibits bone growth *via* the MAPK pathway and reduces chondrocyte proliferation *via* Stat1 (*27*). Another study recently noted a complex pattern of spatial regulation of FGFs and FGFRs (especially FGF2 and FGF4) (*28*), and detailed aspects of this regulatory mechanism must be explored.

2.2. *FGFR3 mutations in the Chinese achondroplastic population*

In 1994, Shiang *et al.* (*18*) confirmed that *FGFR3* was

Table 1. *FGFR3* mutations responsible for achondroplasia

Mutation	Location	Result of mutation	Mutation rate	Type
c.1138G → A	380thExon10	Gly → Arg	≥ 95%	missense
c.1138G → C	380thExon10	Gly → Arg	3-4%	missense
c.1123G → T	375thExon10	Gly → Cys	1-2%	missense
c.1037G → A	364thExon9	Gly → Glu	No data	missense
c.1180A → T*	394thExon10	Thr → Ser	No data	missense
c.649A → T*	217thExon5	Ser → Cys	No data	missense

* new mutation found in the Chinese population.

the gene responsible for achondroplasia for the first time. At about the same time, Bellus *et al*. (*29*) reported a prenatal gene diagnosis of an achondroplastic patient through a villi biopsy, opening up a new area of genetic analysis of *FGFR3* mutations in achondroplasia. In China, research on achondroplasia started only in 2005 when Ma *et al*. (*30*) analyzed the profiles of Chinese patients with achondroplasia for the first time. They used PCR-SSCP to analyze cord blood for *FGFR3* mutations in order to diagnose patients with achondroplasia, providing a potential way to detect or even predict achondroplasia clinically. The same group later (*30*) identified *FGFR3* as a gene responsible for achondroplasia in the Chinese population and they detected the c.1138G→A mutation in Chinese patients. Further investigations have corroborated the finding that most *FGFR3* mutations in the Chinese population are the c.1138G→A mutation; only a handful of studies have noted the c.1138G→C mutation (*31*).

Recently, research on achondroplasia in China has made great progress. Zhu *et al*. (*32*) analyzed probands and families with a history of achondroplasia and found no mutations at the 1138 and 1123 sites, suggesting that a new mutation site may be involved in this pedigree. Further investigation revealed a new mutation, c.1180 A→T at exon 10 (the 394th amino acid), in this pedigree. Using linkage analysis and direct DNA sequence, a study by Zhang *et al*. found that there was a c.649 A→T transition at exon 5 of the *FGFR3* gene in Chinese patients with achondroplasia (*33*). Since it was not present in other normal family members, their finding indicated that this mutation is also pathogenic for achondroplasia. This is the first identified mutation in the Ig II loop of the *FGFR3* gene outside exon 10.

3. Diagnosis of achondroplasia in China

Aging may also play a promoting role in modulating the prevalence of achondroplasia in patients with de-novo gene mutation (*34*), necessitating the development of a precise method of diagnosing the condition in the prenatal stage. To date, several routine methods to diagnose achondroplasia, including ultrasound diagnosis and genetic examination, have been proposed. These methods are quick, efficient, and accurate. In recent years, advanced methods of diagnosing achondroplasia have also been explored. One is the use of high resolution melting (HRM), a new, rapid, and inexpensive method of molecular detection to screen for genetic mutations. He *et al*. (*35*) identified 12 cases, including 10 sporadic cases and 2 cases, in a family with achondroplasia using both HRM analysis and restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR). Of those cases, 11 involved the c.1138G→A heterozygous mutation while 1 involved the c.1138G→C heterozygous mutation. This finding implies that HRM analysis can provide an improved approach over RFLP-PCR in terms of detecting *FGFR3* mutations in patients with achondroplasia.

Denaturing high performance liquid chromatography (DHPLC) is another common method to diagnose achondroplasia in the Chinese population. DHPLC involved the separation and analysis of nucleotide fragments to detect changes in the DNA sequence using a column. In accordance with the melting characteristics of heteroduplex DNA with homologous double-stranded DNA and the fact that heteroduplex DNA has a shorter retention time than homoduplex DNA in the column, the heteroduplex DNA is eluted first, and bimodal or multimodal distributions then yield certain elution curves (*36*). Zhu *et al*. (*37*) detected the pathogenic mutation of *FGFR3* in three families with achondroplasia by DHPLC, restriction enzyme (SfcI and MspI) digestion analysis, and sequencing analysis. They found that all of these methods were able to detect mutations of the *FGFR3* gene although DHPLC is faster, easier, and more sensitive, making it ideal for prenatal genetic diagnosis of patients with achondroplasia.

Achondroplasia can be diagnosed effectively and accurately using the methods mentioned. However, many medical facilities usually combine these methods to confirm a diagnosis of achondroplasia in order to ensure the reliability of the clinical diagnosis and avoid misdiagnosis by a single method.

4. Treatment of achondroplasia in China

4.1. *Hormone therapy*

To date, there are many treatments for achondroplasia (Table 2). In Western countries, growth hormone (GH) therapy has been widely used to lessen the clinical complications of achondroplasia (*38,39*). In China, GH therapy is also a therapeutic option for most patients with achondroplasia. After treatment, some patients improve by becoming taller while others do not, suggesting a patient-specific response to GH therapy. Additionally, the high cost of GH limits the choice of this therapy. For a long time, there have been differing views on the adverse reactions to growth hormone treatment (*30*). A recent follow-up study of patients after 5 years of GH therapy found that GH improved height without any adverse effects on trunk-leg disproportion (*40*).

In addition to GH, a recent study showed that systemic intermittent injection of parathyroid hormone (PTH) may significantly alleviate retarded skeletal development in achondroplastic mice (*41*). In this model, the bone length of the humerus and tibia was extended compared to the bone length in wild-type mice. Furthermore, research has also shown that PTH treatment can alleviate osteopenia and improve bone

Table 2. Current treatment of achondroplasia in China and the West

China	Western countries
Growth hormone (GH)	1. Growth hormone (GH)
Double leg lengthening surgery	2. Osteotomy
	3. Spinal canal decompression
peptide P3	1.Systemic intermittent PTH (1-34)
	2.Selective inhibition of FGFR3 tyrosine kinase
	3. C-type natriuretic peptide
	4. Blocking antibodies to interfere with binding of FGF ligands to FGFR3

structure in achondroplastic mice. At the molecular level, increased PTH-related peptide (PTHrP) expression and down-regulated FGFR3 expression may be responsible for the benefits of PTH in terms of bone growth in achondroplasia, but PTH therapy has not been approved for routine clinical use.

4.2. *Surgical therapy*

Surgical therapy is the most effective treatment option for achondroplasia. In Western countries, a surgical procedure such as osteotomy is often proposed when genu varum is present and persists during childhood. Osteotomy is a preferred surgical treatment for thoracolumbar kyphosis and lumbar stenosis in patients with achondroplasia (*42*). The early experience with surgical limb-lengthening procedures resulted in a high incidence of complications such as pain and infections (*38*), but more advanced procedures have recently resulted in a significant increase in patient height over a 24-month period (*43*).

Spinal canal decompression is one of the most common surgical strategies to treat spinal stenosis in patients with achondroplasia, and it can reduce symptoms of lumbar stenosis (*6*). However, the great risk of neurological injury has discouraged the use of this procedure. The angles and diameters of thoracic and lumbar pedicles of patients with achondroplasia and those of healthy people have been determined despite anatomical differences. Recent studies on the management of spinal disorders in patients with achondroplasia have demonstrated the safety and efficacy of spinal instrumentation (*44,45*).

Double leg lengthening surgery has been proposed as an alternative to treat the Chinese achondroplasia population by restoring the normal ratio of the trunk and lower limbs through extension of the lower limbs (*46*). After surgery, the tibia and femur are extended an average of 10 cm. Since patients with achondroplasia have lower limbs that need to be considerably extended, age is a concern when performing this surgery. Postoperatively, the lower limbs need to be used soon to enhance new bone formation and should be closely monitored for 1-2 years as the epiphyseal plates close.

4.3. *Other potential therapies*

In addition to the methods mentioned earlier, several alternative treatments have recently been proposed to counteract the effects of overactive FGFR3 on endochondral bone formation. One practical example would be use of selective inhibitors of FGFR3 tyrosine kinase, such as imatinib (*47*). Furthermore, the administration of C-type natriuretic peptide (CNP) has also been proposed. Over-expression of CNP in mouse chondrocytes alleviated achondroplasia *via* the MAPK-dependent pathway (*48,49*). In addition, a treatment using blocking antibodies to interfere with FGF-FGFR3 interaction could be another option to treat patients with achondroplasia (*50*). A novel inhibitory peptide for FGFR3 signaling – peptide P3 – was recently identified by a Chinese group (*51*). Peptide P3 exhibits a high binding specificity to the extracellular domain of FGFR3, inhibiting tyrosine kinase activity. It thus may act as a potential therapeutic agent for FGFR3-related skeletal dysplasia.

5. Conclusion

Achondroplasia is a rare autosomal dominant genetic disease that affects many patients in China. Recently, genetic research on achondroplasia in China has made great progress. In China, the condition is being studied and diagnosed *via* ultrasound, a genetic examination, HRM, and DHPLC and treated *via* GH therapy, double leg lengthening surgery, and peptide P3. However, the study of achondroplasia is still in its infancy and its pathogenesis is unclear, resulting in a lack of effective treatments. As molecular genetic techniques develop, the pathogenesis of this condition will be studied and more effective treatments are anticipated for patients with achondroplasia in China.

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Review

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Melorheostosis and a review of the literature in China

Chi Zhang[1], Wenda Dai[1], Yi Yang[1], Qi Tang[2], Zhenjun Yao[1,*]

[1] *Orthopaedic Department, Zhongshan hospital, Fudan University, Shanghai, China;*
[2] *Department of Health Care Management and Maternal and Child Health, Shandong University, Ji'nan, Shandong, China.*

Summary **Melorheostosis is an uncommon, non-genetic, non-developmental, sclerosing dysplasia of bone and adjacent soft tissues, with deformity of the extremity, pain, limb stiffness and limitation of motion. The characteristic radiographic appearance consists of irregular hyperostotic changes of the cortex resembling melted wax dripping down the side of a candle. In this review, clinical characteristics of Melorheostosis are discussed and reports in the Chinese literature are summarized.**

Keywords: Melorheostosis, sclerosing bone dysplasia, developmental anomalies, bone sclerosis, China

1. Introduction

Melorheostosis is a rare, non-hereditary, benign, sclerosing mesodermal dysplasia which affects the skeleton and adjacent soft tissues (*1-3*). It got this name because of the characteristic periosteal hyperostosis along the cortex of long bones which looks similar to the flowing or dripping of candle wax (originated from Greek, melos = limb, rhein = to flow, ostos = bone) (*4-6*). Melorheostosis is also known by other synonyms such as candle disease of the bone, and osteosis eburnisans monomelica (*7,8*). Until now, there have been about 400 cases reported in the English literature (*9*).

In 1922 Melorheostosis was first described by Leri and Joanny, and was also called Leri's disease or syndrome thereafter (*10*). This condition may affect only one bone (monostotic form, representing a forme fruste of the disorder), one limb (monomelic form), or multiple bones (polyostotic form). Although a benign dysplasia, the osseous changes can cause morbidity. Skin and subcutaneous tissue involvement can result in fibrosis and joint contractures leading to deformity and limb-length discrepancy (*3,11-13*).

The aim of this study is to summarize the classical features of this disease and review the literature reported in China, to enrich our knowledge of melorheostosis and provide information in the Chinese population, which we believe will enhance understanding of this anomaly and improve the accuracy of diagnosis and efficacy of treatment.

2. Search strategy

The electronic database of Chinese Medicine, Wanfang Data, was searched using the keyword "melorheostosis" to identify all literature published in peer-reviewed Chinese journals since January 1990. The full text was reviewed and clinically related data extracted, summarized and discussed. Cases from the same authors were examined to avoid repetition.

3. Etiologiy and pathogenesis

The etiology of melorheostosis remains unknown. There have been various theories proposed to explain the pathogenesis of this disease such as a developmental disorder theory (*14*), ischemic theory (*15*), telangiectatic theory (*16*), and infective theory (*17*). Currently, there are two major hypotheses in existence. In 1979, Murray and McCredie (*18*)correlated melorheostosis with sclerotomes, hypothesizing that melorheostosis might be the result of a segmental sensory lesion due to a specific infection, insult, or injury to segments of the neural crest during embryogenesis, which partially explains the peculiar monomelic involvement of melorheostosis. In 1995, Fryns (*19*) proposed mosaicism to explain the sporadic occurrence of dysplasia which suggests

Zhang C and Dai WD contributed equally to this work.

**Address correspondence to:*
Dr. Zhenjun Yao, Orthopaedic department, Zhongshan hospital, Fudan University, Shanghai 200032, China.
E-mail: yao.zhenjun@zs-hospital.sh.cn

that the asymmetric involvement of skeletal structures and concomitant vascular and hamartomatous changes in the overlying soft tissues result from an early post-zygotic mutation of the mesenchyme which explains why the extent of involvement is so variable and why the incidence ratio in both genders is equal.

4. Histopathology

As has been reported by various researchers, microscopic examination of cortical specimens from melorheostosis patients reveals nonspecific periosteal bone formation with thickened trabeculae and fibrotic changes in the marrow spaces (*1-3*). These bones consist mostly of primary haversian systems and are largely obliterated by the deposition of sclerotic, irregular, and thickened lamellae, particularly on the periosteal surface (*4,6,20*).

Islands of cartilage have been described in periarticular lesions with evidence of endochondral bone formation in addition to intramembranous bone formation within the peri-joint cellular fibrous tissue (*4*). Osteoblastic activity along the margins of osteons is common, while osteoclastic activity is never a prominent feature although occasionally noted (*20*).

5. Clinical Presentation

Onset of melorheostosis is usually insidious. The symptoms, which include pain, limb stiffness, limitation of motion in the joints, and deformity of the involved extremity, usually do not manifest until late childhood or early adolescence and tend to progress into adult life (*1-4*). The disease usually exhibits a chronic course with periods of exacerbation and arrest. Progression of melorheostosis can be rapid in childhood but often slower in adulthood, during which joint stiffness and pain are the predominant symptoms (*6-8,10,12,21-26*). Because the abnormal ossifications frequently involve soft tissues and extend into the joints, the latter often exhibit a restricted range of motion as the result of contracture and fibrosis. Other deformities are also common, including flexion contractures of the hips and knees, varus or valgus deformities of the feet, and overlapping toes (*27-29*). Joint ankylosis may be present as a result of heterotopic bone formation and soft-tissue calcification (*30,31*). Laboratory findings for serum calcium, phosphorus, and alkaline phosphatase levels have been reported to be within normal limits (*1-3,6*).

6. PrP^C: a novel therapeutic target for AD

Flowing cortical hyperostosis along one side of the shaft of the long bone resembling "melting wax flowing down the side of a candle" is the characteristic radiographic appearance of melorheostosis(*2-4,28,31-34*). The areas of dense hyperostosis have a linear, segmental distribution and tend to extend distally from one bone to the next, ultimately involving the bones of one or more digits. The classic presentation is not always seen in all patients and there are other patterns well described by Freyschmidt (*6*). These include: *i*) osteoma-like appearance with hyperostosis located either on the outer or inner aspect of the affected bone (the most common pattern in his study); *ii*) osteopathia striata-like pattern, which shows long and dense hyperostotic striations near the inner side of the cortex in two or more bones. Occasionally, melorheostosis may be mistaken for osteopathia striata, but the striations in melorheostosis are much larger, broader and unilateral, unlike the genuine osteopathia striata; *iii*) myositis ossificans-like ossifications in soft tissues, which are more nodular in arrangement without any lamellar appearance to the ossification. In later stages of the disease, endosteal hyperostosis may be seen, and this can partially or completely obliterate the medullary cavity. Bony overgrowth, particularly around the hip, may simulate osteochondroma.

7. Diagnosis and differentiation diagnosis

The polystotic form of melorheostosis, due to its characteristic appearance on conventional radiography, can be readily distinguished from other lesions (*35,36*). However, monostotic lesions can have varied appearances, and should be differentiated from myositis ossificans, osteochondroma, osteoid osteoma and parosteal osteosarcoma (*37,38*). In myositis ossificans the ossification is more significant at the periphery than at the center, and a radiolucent cleft can be seen between the lesion and the cortex. Mature myostitis ossificans usually shows no uptake or only minimal activity on scintigraphy. In osteoid osteoma, unlike melorheostosis, the surface of the lesion is usually smooth rather than wavy, and a confident diagnosis can be made when the nidus is observed on cross-section imaging. In osteochondroma, the cortex of the lesion is continuous with the cortex of the parent bone and there is also continuity of the medullary cavity, which are its characteristic features, while in melorheostosis, the lesion typically has paraosteal or endosteal involvement. In parosteal osteosarcoma, the major sign to differentiate it from melorheostosis is bone destruction. Radionuclide bone scintigraphy can also help with intense uptake and increased activity in the medullary cavity.

8. Treatment

Various conservative or surgical methods have been practiced in treating the pain and deformities associated with melorheostosis. Conservative therapies include oral medications such as bisphosphonates, NSAIDs, and

nifedipine. Other nonsurgical treatments have also been used such as physical therapy, manipulations, serial casting, braces, and nerve block, but unfortunately most of these methods are ineffective. Surgical procedures consist of soft-tissue procedures such as tendon lengthening, excision of fibrous and osseous tissue, fasciotomy, capsulotomy, osteotomies, excision of hyperostoses, arthrodesis, contralateral epiphysiodesis, and amputation (*1-6,39-41*). When treating limb deformities associated with surgical treatments in melorheostosis patients, recurrences are common.

9. Chinese literature review and discussion

As a result of the Chinese literature review, 104 papers were retrieved from the database since January 1990, among which 95 were case reports and 9 case series studies. The total number of Melorheostosis cases reported in these last 23 years in China is 223. The male/female ratio in these cases was 115/108. The age at which the cases were diagnosed ranges from 3 to 72. Though the etiology of Melorheostosis remains unknown, research indicates that women and men are affected equally by the disorder, and it usually presents after early childhood. As was reported in the English literature, Melorheostosis occurs at about 0.9 cases per million population and is a very uncommon disease (*9*). But unfortunately, we failed to find any study that investigated the incidence of melorheostosis in the Chinese population.

Melorheostosis's onset is often insidious. In our study, 14 patients out of 223 (6.28%) were found accidentally. 3 of which were diagnosed by routine physical examination, and the other 11 by regular imaging because of trauma in the ipsilateral limb. The most common complaints of the symptomatic patients in our study were limb pain, joint stiffness, limitation of joint motion, and later on deformity.

The distribution of the lesion is mostly segmental and unilateral (hemimelic). It may affect only one bone (monostotic), one limb (monomelic) or multiple bones (polyostotic), which in our study, were 3.14%, 78.53% and 18.33% respectively. In cases with multiple adjacent bones affected, the lesions tend to be in a sclerotomal distribution, and the lower extremity is more frequently involved than the upper extremity. The long tubular bones are most commonly affected although the disease may involve any of the short bones of the hand and foot. Melorheostosis can also have rare features such as spinal involvement, para-articular soft-tissue masses and intra-articular extensions. In our cases, 2 patients were found with lumbar spine involovement, but with no neurogenic symptoms (*26,42*). The co-occurrence of melorheostosis with osteopoikilosis was presented in 3 cases (*43,44*).

The spectrum of diseases might be very different between countries and cultures. As rare as melorheostosis is, there are still clues leading to a correct diagnosis of uncommon cases. We hoped to provide evidence that might not be easily accessed by non-Chinese physicians and share our experience with foreign doctors with this review.

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Brief Report

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Serum levels of leptin receptor in patients with systemic sclerosis

Yukimi Ohyoshi, Takamitsu Makino*, Masatoshi Jinnin, Wakana Nakayama, Satoshi Fukushima, Yuji Inoue, Hironobu Ihn

Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan.

Summary **Microvascular damage is one of the primary pathologic components of systemic sclerosis (SSc). Serological abnormalities of angiogenic and angiostatic factors in SSc have previously been described. Like these factors, the plasma levels of leptin were significantly elevated in patients with SSc in comparison to normal controls. However, leptin receptor has not been examined in patients with SSc. The current study used sandwich ELISA to evaluate the serum levels of leptin receptor in patients with SSc. Serum samples were obtained from 36 patients with SSc. Samples were also obtained from 12 healthy control subjects and 10 patients with scleroderma spectrum disorder (SSD) who did not fulfill the criteria for SSc but who had the potential to develop SSc. Mean serum leptin receptor levels were significantly higher in patients with SSD than in patients with SSc (255.7 ng/mL *vs.* 184.6 ng/mL, $p < 0.05$ according to a *Mann-Whitney* test). There were no statistically significant differences between healthy control subjects and patients with SSc. Clinical parameters were evaluated, and the frequency of esophageal reflux was significantly lower in patients with elevated serum leptin receptor levels than in those with reduced levels (6.3% *vs.* 35.3%, $p < 0.05$). In summary, these results suggest that the serum levels of leptin receptor are a clinically useful marker of SSD, and measurement of serum leptin receptor over time in patients with SSD may lead to early detection of SSc.**

Keywords: Leptin receptor, systemic sclerosis, scleroderma spectrum disorder, ELISA

1. Introduction

Systemic sclerosis (SSc), or scleroderma, is an acquired disorder that typically results in fibrosis of the skin and internal organs. Although the pathogenesis of SSc is still unclear, it includes inflammation, autoimmune attack, and vascular damage. The condition leads to the activation of fibroblasts and abnormal accumulation of extracellular matrix, mainly in the form of type I collagen (*1,2*).

Vascular damage is one of the primary pathologic components of SSc. Raynaud's phenomenon, or aberrant nailfold bleeding, is known to be an early vascular event associated with this disease. Telangiectasias, pitting scars, skin ulcers, impaired wound healing, and pulmonary hypertension are frequently observed in the disease process and can severely affect the quality of life of these patients. Serological abnormalities of angiogenic and angiostatic factors, including vascular endothelial growth factor, angiopoietin-2, and platelet-derived growth factor, in SSc have previously been described; uncontrolled activation of such signaling rather than its inactivation may be the cause of the disturbed vessel morphology in sclerotic skin (*3,4*).

Significantly increased plasma levels of leptin were also reported in patients with SSc in comparison to normal controls (*5*). Leptin, the *ob* gene product consisting of 146 amino acid residues, is known to be secreted by adipocytes (*6*). Leptin helps to regulate body weight by affecting food intake, energy expenditure, and thermogenesis (*7*). Furthermore, leptin is involved in many physiological processes, including angiogenesis, by stimulating endothelial cell proliferation (*8*).

Leptin takes action by binding to its receptor. The leptin receptor usually consists of an extracellular domain and cytoplasmic portion and is restricted to the cell surface. Leptin receptor levels are highest in

**Address correspondence to:*
Dr. Takamitsu Makino, Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan.
E-mail: takamitu-makino@fc.kuh.kumamoto-u.ac.jp

infants, decrease into adolescence, and remain stable throughout adulthood (*9*). The receptor is expressed predominantly in areas of the hypothalamus, indicating that the leptin receptor also plays an important role in regulating body weight (*7*). That said, the receptor is also associated with conditions that negatively affect health. The extracellular domain of the receptor can be secreted into body fluid in soluble form. Soluble leptin receptor is found to be up-regulated in patients with obesity as well as in chronic heart failure, end-stage renal disease, and anorexia (*10*). However, serum leptin receptor levels have not been examined in patients with rheumatic diseases.

The current study posited that leptin signaling contributes to the pathogenesis of vascular damage in SSc and it sought to evaluate the potential for serum levels of leptin receptor to be a useful marker of SSc.

2. Materials and Methods

2.1. *Clinical assessment and patient characteristics*

The rheumatic diseases of systemic lupus erythematosus (SLE), dermatomyositis (DM), and SSc as are associated with vasculopathy or dysfunction of endothelial cells were studied. Patients with SSc or SLE fulfilled the criteria proposed by the American College of Rheumatology (ACR) (*11,12*). SSc was categorized as diffuse cutaneous SSc (dcSSc) or limited cutaneous SSc (lcSSc) according to the classification system proposed by LeRoy *et al*. (*13*). The concept of scleroderma spectrum disorder (SSD) was originally proposed by Maricq *et al*. to unify typical SSc, early forms of SSc, and closely related disorders, including mixed connective tissue disease (MCTD) (*14,15*). Ihn *et al*. later redefined SSD as patients did not fulfill the criteria for SSc but some later developed SSc, so they suggested a new method of diagnosis using a point system to distinguish SSD from early SSc. A total score was obtained as the sum of the following five factors: *i*) extent of skin sclerosis (maximum, 10 points), *ii*) pulmonary changes (maximum, 4 points), *iii*) antinuclear antibodies (maximum, 5 points), *iv*) pattern of Raynaud's phenomenon (maximum, 3 points), and *v*) nailfold bleeding (maximum, 2 points). A score of 9 or more points is consistent with SSc and a score of 5 to 8 points is consistent with SSD (*16*). Patients diagnosed with SSD who fulfilled the criteria proposed by Ihn *et al*. (*16*) were also included in the current study. Classical DM was diagnosed based on the criteria proposed by Bohan and Peter (*17*). Clinically and histopathologically typical cutaneous lesions without classical myositis were diagnosed as clinically amyopathic DM (CADM) in accordance with previous criteria (*18,19*). Clinical and laboratory data reported in the current study were obtained at the time of serum sampling.

2.2. *Measurement of leptin receptor levels*

Levels of serum leptin receptor were measured with a specific ELISA kit (Human leptin receptor, BioVendor Laboratory Medicine, Czech Republic) (*20*). Briefly, monoclonal anti-human leptin receptor antibodies were precoated onto microtiter wells. Aliquots of serum were added to each well, followed by peroxidase-conjugated antibodies against leptin receptor. Color was developed with hydrogen peroxide and tetramethylbenzidine peroxidase and absorbance at 450 nm was measured. Wavelength correction was performed based on absorbance at 630 nm. The level of leptin receptor in each sample was determined by interpolation from a standard curve.

2.3. *Statistical analysis*

Statistical analysis was carried out with a *Mann-Whitney* test for the comparison of median, and Fisher's exact probability test for the analysis of frequency. A p less than 0.05 was considered significant.

3. Results and Discussion

3.1. *Serum levels of leptin receptor in patients with SSc*

Serum leptin receptor levels in patients with various rheumatic diseases and in healthy control subjects are shown in Figure 1. Serum samples were obtained from 36 patients with SSc (13 dcSSc and 23 lcSSc). Samples were also obtained from 12 healthy control subjects, 10 patients with SLE, 15 patients with DM, 5 patients with CADM, and 10 patients with SSD who did not fulfill the criteria for SSc but who had the potential to develop SSc. Patients with diabetes, obesity, atherosclerosis, or metabolic syndrome and those who had been treated were excluded.

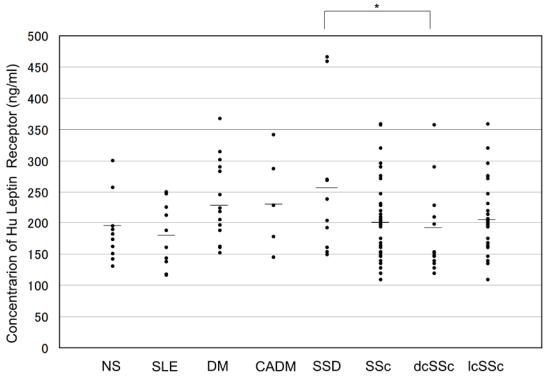

Figure 1. Serum concentrations of leptin receptor in patients with SSc, SSD, SLE, classical DM, CADM, and NS. SSc was classified as dsSSc or lcSSc. Serum leptin receptor levels were measured with an ELISA kit as described in materials and methods. Serum leptin receptor concentrations are shown on the ordinate. Bars indicate means. A p less than 0.05 is considered significant.

Mean serum leptin receptor levels were highest in patients with SSD. Although mean leptin receptor levels were elevated in patients with SSD compared to those in patients with SSc or in healthy control subjects, there were no statistically significant differences among the groups. Mean serum leptin receptor levels were significantly higher in patients with SSD than those in patients with dcSSc (255.7 ng/mL *vs*. 184.6 ng/mL, $p < 0.05$). Serum leptin receptor levels may transiently increase in the SSD stage but normalize in the SSc stage. Because progressive tissue fibrosis caused by SSc is often irreversible, at least clinically, new strategies need to be quickly developed to diagnose patients as early as possible and follow them closely. Accordingly, the concept of SSD should be better understood and characterized. The current findings suggest that elevated serum leptin receptor levels may serve as a useful marker for the differentiation of SSD from SSc. Moreover, patients with SSD frequently have an increased risk of developing SSc in the future. Measurement of serum leptin receptor levels over time in patients with SSD may lead to early detection of SSc. The current study did have one limitation in that it involved a small sample of patients with SSD because SSD is a fairly rare disease. However, the current approach may be a useful way to diagnose SSD. Studies with larger samples are needed in the future.

3.2. *Correlation of serum leptin receptor levels with clinical and serological features of SSc*

Table 1 shows the clinical and laboratory features of SSc in conjunction with elevated or reduced leptin receptor levels. There were no significant differences between these two groups in term of sex, age of onset, or the prevalence of dcSSc. However, esophageal reflux was significantly less prevalent in patients with elevated serum leptin receptor levels than in those with reduced levels (6.3% *vs*. 35.3%, $p < 0.05$). As noted earlier, leptin receptor was thought to possibly be involved in the pathogenesis of vascular abnormalities in patients with SSc, but serum levels were not associated with the prevalence of Raynaud's phenomenon, pitting scars, nailfold bleeding, or pulmonary hypertension. Esophageal reflex is treatable and reversible but cannot be detected by serology. Serum leptin receptor may serve as a clinically useful marker. Since patients with SSD had higher leptin receptor levels than both patients with SSc and control subjects and since patients with SSc and elevated serum levels had less esophageal reflux, leptin receptor levels may increase in the very early stage of this disease and thus act to mask the SSc phenotype. However, the exact role of leptin receptor in the pathogenesis of SSc remains unclear. Further studies are needed to clarify these aspects.

In conclusion, mean serum leptin receptor levels were highest in patients with SSD. These results suggest that elevated serum leptin receptor levels may serve as a useful marker for the differentiation of SSD from SSc and the reduced prevalence of esophageal reflex in SSc.

Table 1. Correlation of serum leptin receptor levels with clinical and serological features in patients with systemic sclerosis (SSc)

Items	Serum leptin receptor levels	
	Elevated (n = 18)	Low (n = 18)
Age at the time of serum sampling (mean years, interquartile range)	63.7	59.5
Duration of disease (mean years, interquartile range)	102	41.3
Type (diffuse: limited)	6:11	8:09
MRSS (point)	8.4	13.17
Clinical features		
Pitting scars/ulcers	41.2	50
Nailfold bleeding	58.8	66.7
Raynaud's phenomenon	88.2	92.9
Telangiectasia	25	21.4
Contracture of phalanges	85.7	85.7
Calcinosis	0	12.5
Diffuse pigmentation	36.4	20
Short SF	69.2	81.8
Sicca symptoms	53.9	53.9
Organ involvment		
Pulmonary fibrosis	29.4	44.4
Mean % VC (%)	105	97.6
Mean % DLco (%)	89.6	84.2
Pulmonary hypertension	27.9	28.3
Oesophagus	6.3*	35.3
Heart	37.5	17.7
Kidney	0	0
Joint	50	66.7
Thrombosis	0	0
ANA specificity		
Anti-topoI	22.2	33.3
Anti-centromere	50	27.8
Anti-U1 RNP	16.7	16.7

Unless indicated, values are percentages. MRSS: Modified rodnan total skin thickness score; SF: Sublingual frenulum; VC: Vital capacity; DLco: Diffusion capacity for carbon monooxidase; ANA: Antinuclear antibodies; Anti-topo I: Anti-topoisomerase I antibody; Anti-centromere: Anti-centromere antibody. *$p < 0.05$ versus patients with normal leptin receptor levels according to a *Mann-Whitney* test.

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Original Article

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A comparative proteomics study on matrix vesicles of osteoblast-like Saos-2 and U2-OS cells

Liang Jiang[1,2], Yazhou Cui[1], Jing Luan[1], Xiaoyan Zhou[1], Xiaoying Zhou[1], Jinxiang Han[1,*]

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

Summary **Matrix vesicles (MVs) play an important role in the initial stage of the process of bone mineralization, and are involved in multiple rare skeletal diseases with pathological mineralization or calcification. The aim of the study was to compare the proteomic profiling of osteoblast-like cells with and without mineralization ability (Saos-2 and U2-OS), and to identify novel mineralization-associated MV proteins. MVs were extracted using ExoQuick solution from mineralization-induced Saos-2 and U2-OS cells, and then were validated by transmission electron microscopy. A label-free quantitative proteomic method was used to compare the protein profiling of MVs from Saos-2 and U2-OS cells. Western-blots were used to confirm the expression of MVs proteins identified in proteomic studies. In our proteomic studies, we identified that 89 mineralization-related proteins were significantly up-regulated in Saos-2 MVs compared with U2-OS MVs. We further validated that two MVs proteins, protein kinase C α and ras-related protein Ral-A, were up-regulated in MVs of Saos-2 cells compared to those of U2-OS cells under mineralization-induction. Our findings suggest that protein kinase C α and ras-related protein Ral-A might be involved in bone mineralization as MVs components.**

Keywords: Matrix vesicle, osteoblasts, mineralization, proteomics

1. Introduction

Matrix vesicles (MVs) are small vesicles with a diameter of 50-200 nm, which contain abundant phospholipid and protein components and play an important role in the process of bone mineralization (*1*). It has been reported that a MV protein deficiency might participate in the pathological mineralization process of many rare skeletal diseases (*2*). Recently, the protein profiling of MVs from different origins were analyzed in several proteomic studies, and more than 2,000 proteins have been identified in MVs including several validated mineralization-related proteins such as alkaline phosphatase (TNAP), and annexins (*3-6*). However, the roles of most of the MV proteins in mineralization regulation remain unclear.

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

Saos-2 is a human osteoblast-like cell line having significant mineralization ability under osteogenic induction. Meanwhile, another human osteoblast-like cell U2-OS has low phosphate enzyme activity and could not be mineralized after induction (*7*). Therefore, we speculated that the difference among MV proteins between Saos-2 and U2-OS cells could provide novel clues to clarify the exact roles of MVs during the mineralization process.

In this study, we performed a label-free LC-MSMS proteomic approach to compare the MV protein profiles between Saos-2 and U2-OS cells in order to further screen functional MV proteins for mineralization regulation.

2. Materials and Methods

2.1. *Cell culture and osteogenic induction*

Human Saos-2 and U2-OS cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). U2-OS and

Saos-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone, Thermo Scientific, Logan, UT, USA) and McCoy's 5A Medium (Gibco, Life Technologies, Carlsbad, CA, USA) respectively, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Beyotime, Haimen, Jiangsu, China) at 37°C under 5% (v/v) CO_2 in a humidified atmosphere. Mineralization was induced on confluent cells in induction medium supplemented with 50 μg/mL L-ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) and 10 mM β-glycerophosphate (Sigma-Aldrich).

2.2. *Mineralization detection by Alizarin Red staining*

Saos-2 and U2-OS cells were plated in 24-well plates for induction, and mineralization levels were assessed by alizarin red staining during induction (0 days, 3 days, and 7 days). The cells were washed with PBS and fixed with 4% paraformaldehyde. Then the cells were washed with PBS and stained with 0.5% (w/v) alizarin red S solution for 1 h. After washing with PBS, the stained cultures were photographed.

2.3. *Matrix vesicles extraction by ExoQuick solution*

Cells were washed with PBS without calcium-magnesium ions 3 times, and then collagenase (2 mL, 1 mg/mL) was added. Supernatant was collected and centrifuged at 3,000 rpm for 30 min at 4°C, transferred into a 100 kD ultrafiltration concentrator tube (Millipore, Billerica, MA, USA) and centrifuged at 3,000 rpm for 30 min at 4°C to concentrate to about 1 mL. The concentrated liquid was mixed with the ExoQuick Exosome Isolation Reagent (System Biosciences, Mountain View, CA, USA) at 4°C overnight. The mixture was centrifuged at 3000 rpm for 30 min at 4°C to sediment MVs.

2.4. *Transmission electron microscopy validation for matrix vesicles*

Freshly isolated MVs pellets were first fixed with 3% glutaraldehyde at 4°C for 2 h, and then post-fixed in 1% osmium tetroxide for 1 h. After dehydration in a graded ethanol series with acetone, samples were embedded in epoxy resin. 75 nm thick semithin sections were mounted on copper grids, and stained with uranyl acetate while lead citrate solutions were used to enhance the contrast. Electron micrographs were observed on a H800 transmission electron microscope (TEM) (Hitachi Electronic Instruments, Tokyo, Japan).

2.5. *LC-MS/MS analysis and label-free quantification*

MVs protein samples were extracted by adding 200 μL SDT buffer containing 4% SDS, 150 mM Tris (pH 8.0). After heating and sonication, the lysates were centrifuged (10,000 g, 1 h, 4°C) and the supernatants were collected. The protein concentrations were assayed with a standard Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). 150 μg of MVs protein samples from U2-OS and Saos-2 cells (with and without mineralization induction) were digested by a filter-aided sample preparation (FASP) method as described previously (*8*). Mass analyses were performed on a Q Exactive mass spectrometer. MS/MS spectra were searched against the nonredundant Inter-national Protein Index (IPI) human protein database or Swiss-prot database. Label-free quantitative analyses were performed as described previously (*9*). MaxQuant output files were uploaded into a Perseus package for calculation of significant scores. Proteins with scores that varied more than 2 fold were defined as significantly differentially expressed.

2.6. *Western-blots*

Equal amounts (25 μg) of MVs protein samples were separated on a 12% SDS-PAGE gel, and transferred to PVDF membranes (Millipore), and then probed with primary antibodies against protein kinase C α (Ptglab, 1:1000 dilution) and ras-related protein (Ptglab, 1:1000 dilution) overnight at 4°C, followed by peroxidase-conjugated secondary antibodies. Finally, the bands were visualized using ECL reagents (Millipore) according to the manufacturer's instructions.

3. Results

After osteogenic induction, Saos-2 cells demonstrated a time-dependent increase in mineralized nodule formation assessed by Alizarin Red staining, while mineralization was absent in the matrix of U2-OS cells (Figure 1). Using ExoQuick reagents, MVs were successfully precipitated, and were validated by

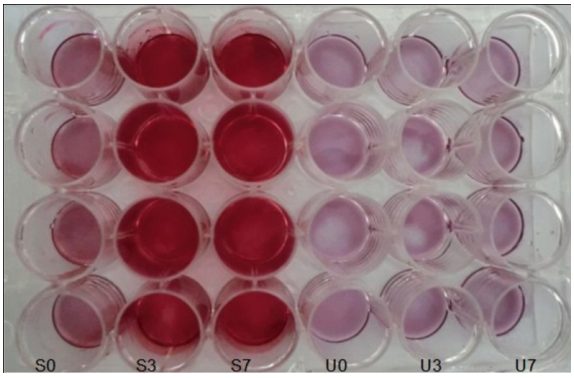

Figure 1. Alizarin Red test for the mineralization of U2-OS and Saos-2 cells. Saos-2 cells for 0 day, 3 days and 7 days after mineralization induction, respectively (S0,3,7); U2-OS cells for 0 day, 3 days and 7 days after mineralization induction, respectively (U0,3,7). Saos-2 cells appear significantly mineralized after induction for 3 days, while in U2-OS cells mineralization was still not clear after induction for 7 days.

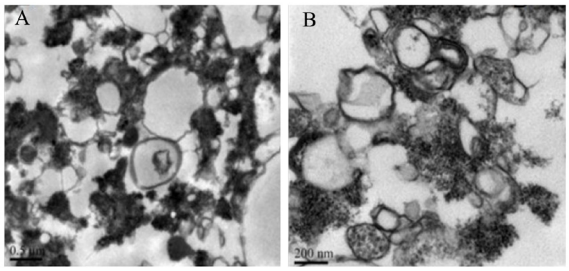

Figure 2. Representative images of Transmission electron microscopy on MVs. (A) Saos-2 MVs with mineralization nodules after induction for 7 days; **(B)** U2-OS MVs without mineralization nodules after induction for 7 days.

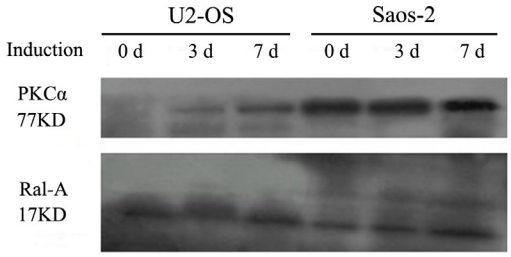

Figure 3. Western-blot analysis on protein kinase C α (PKCα) and ras-related protein Ral-A on MVs from Saos-2 and U2-OS cells at different induction times. Saos-2 MVs had higher PKCα expression levels than U2-OS MVs; Saos-2 MVs showed additional bands of Ral-A.

Table 1. Top list of GO molecular function on up-regulated MVs proteins in Saos-2 cells compared with U2-OS cells

GO Term	Count	*p*-Value	*q*-Value
GO:0005515 protein binding	51	7.16E-51	1.15E-48
GO:0000166 nucleotide binding	34	3.84E-46	3.09E-44
GO:0005524 ATP binding	20	2.44E-26	1.07E-24
GO:0005525 GTP binding	13	1.98E-22	6.84E-21
GO:0005509 calcium ion binding	12	1.68E-15	4.51E-14
GO:0003924 GTPase activity	10	3.88E-19	1.25E-17
GO:0016491 oxidoreductase activity	10	9.55E-14	2.00E-12
GO:0051082 unfolded protein binding	8	1.08E-16	3.08E-15
GO:0003779 actin binding	7	3.66E-11	6.09E-10

Table 2. Top list of GO molecular function on up-regulated MVs proteins in Saos-2 cells compared with U2-OS cells

GO Term	Count	*p*-Value	*q*-Value
GO:0044419 interspecies interaction between organisms	26	1.04E-53	2.50E-51
GO:0002474 antigen processing and presentation of peptide antigen *via* MHC class I	19	4.73E-48	5.71E-46
GO:0006955 immune response	19	5.59E-28	3.00E-26
GO:0019882 antigen processing and presentation	18	3.59E-40	2.48E-38
GO:0015031 protein transport	11	9.69E-15	2.46E-13
GO:0055114 oxidation reduction	10	4.31E-14	9.91E-13
GO:0007264 small GTPase mediated signal transduction	8	1.76E-11	3.14E-10
GO:0007165 signal transduction	8	6.27E-05	3.74E-04
GO:0008152 metabolism	8	0.009911	0.015593

transmission electron microscopy. As seen in Figure 2, the precipitation by ExoQuick reagents recognized spherical membrane-bound vesicle structures with diameters ranging from 50 to 200 nm, and part of the vesicles contained electron dense material.

In the proteomic study, we identified a total of 175 differentially expressed proteins (fold > 2) in Saos-2 MVs compared with U2-OS MVs, including 89 up-regulated proteins and 86 down-regulated proteins (Supplemental data, Table 1S (*http://www.irdrjournal.com/docindex.php?year=2013&kanno=2*)). Among the up-regulated MVs proteins, alkaline phosphatase (ALP) ranked as the most significantly increased MVs protein in Saos-2 cells, which has been proved in many studies. 89 up-regulated proteins were further classified on Gene Ontology in terms of biological processes (Table 1) and molecular function (Table 2) using the MAS 3.0 software (CapitalBio, Beijing, China). In particular, we observed that 12 up-regulated MVs proteins of Saos-2 cells belong to calcium ion binding proteins (GO: 0005509).

To confirm the results of our proteomic study, two up-regulated MV proteins in Saos-2 cells (protein kinase C α and ras-related protein Ral-A) were selected for validation by Western blotting. We detected that both MVs proteins were overexpressed in Saos-2 cells compared to U2-OS cells, which is consistent with our proteomic findings (Figure 3).

4. Discussion

Matrix vesicles have been implicated in pathological mineralization events, which are characteristic of multiple rare diseases. In this study, we compared the MVs protein profiles between mineralization-competent cells Saos-2 and mineralization incompetent cells U2-OS. We identified a panel of differentially expressed proteins at MVs levels associated with mineralization, which could provide novel clues for mining the regulatory details of mineralization.

We performed a GO analysis on 89 up-regulated MV proteins in Saos-2 cells. In molecular function analysis, differentially expressed proteins were mainly

categorized into protein binding, nucleotide binding, ATP-binding, GTP-binding and calcium ion binding groups. In particular, a subgroup of calcium ion binding proteins were identified including mannose receptor [C type 2], protein kinase C [alpha] (PKCα), profilin 1, heat shock protein 90 kDa beta, macrophage migration inhibitory factor, S100 calcium binding protein A6, annexin A6, NAD-dependent malic enzyme [mitochondrial], S100 calcium binding protein A13, and transketolase. Most of these proteins have been proved to be involved in mineralization process regulation, such as, annexin A6 (*10,11*). However, several calcium ion binding proteins identified in this study have not been reported. We selected one of them, protein kinase C α (PKCα), for further validation. Our Western-blot study confirmed that Saos-2 MVs had an increased expression level of PKCα compared to U2-OS during mineralization induction. Previous studies indicated that PKCα plays an important role in osteoblast differentiation and mineralization. Bawden *et al*. (*12*) found that PKCα was localized in differentiating odontoblasts and the PKC signal transduction pathway may be involved in key inductions in the early stages of dentin and enamel formation. Miraoui *et al*. (*13*) demonstrated that PKCα acts as an important regulator in FGFR2-induced osteogenic differentiation of mesenchymal cells. Our data for the first time found that PKCα could also be located in MVs and up-regulated in mineralization-competent cells which suggests PKCα might be engaged in the mineralization process at the MVs level, however, its exact mechanism needs further investigation.

Phospholipase D is an important enzyme for the mineralization process (*14*). Phospholipase D is engaged in a high rate of hydrolysis of neutral phospholipids and a lower rate of degradation of anionic phospholipids, which allows mineral formation in MVs (*15*). It has been proposed that ras-related protein Ral-A is involved in the tyrosine kinase-mediated, Ras-dependent activation of phospholipase D (*16*). In our study, we confirmed that Ral-A also exists as a MVs component, and suggests that its interaction with phospholipase D in MVs might participate in the regulation of the mineralization process.

In conclusion, in this study we identified a series of MVs proteins specifically up-regulated in mineralization competent osteoblasts, which might provide new clues to study the mechanism for mineralization control in the extracelluar matrix. We also confirmed that protein kinase C α and ras-related protein Ral-A are novel MVs proteins and might be involved in bone mineralization as MVs components.

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Case Report

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Clinicopathological subclassification of biliary cystic tumors: Report of 4 cases with a review of the literature

Suguru Yamashita[1,*], Nobutaka Tanaka[1], Michiro Takahashi[1], Shojiro Hata[1], Yukihiro Nomura[1], Kenji Ooe[2], Yoshio Suzuki[2]

[1] *Department of Surgery, Asahi General Hospital, Chiba, Japan;*
[2] *Department of Pathology, Asahi General Hospital, Chiba, Japan.*

Summary **Biliary cystic tumors are rare hepatic neoplasms, and knowledge regarding the origin and pathology of these tumors remains vague. They should be analyzed in more detail. In our institution, 4 biliary cystic tumor surgeries were performed between December 1999 and March 2010. Pathological evaluation of resected specimens was performed to evaluate the characteristics of the intracystic epithelium and to determine the presence or absence of interstitial infiltrate, ovarian mesenchymal stroma (OMS), luminal communication between the cystic tumor and the bile duct, and mucin (MUC) protein expression. We evaluated the following 4 cases: case 1, a 21-year-old woman with a biliary cystadenoma who underwent extended right hepatectomy; case 2, a 39-year-old woman with a biliary cystadenoma who underwent left hepatectomy; case 3, an 80-year-old man with a biliary cystadenoma who underwent left hepatectomy; and case 4, a 61-year-old man with a biliary cystadenocarcinoma revealing papillary proliferation of atypical epithelium and interstitial infiltrates who underwent left hepatectomy. Case 3 had papillary proliferation of the intracystic atypical epithelium but showed interstitial infiltrates. Luminal communication with the bile duct, centrally or peripherally, was found in all 4 cases. Only case 2 showed OMS. Immunohistochemical staining revealed the following findings: cases 1 and 2, MUC1-/MUC2-; case 3, MUC1+/MUC2-; and case 4, MUC1+/MUC2+. It is important to gather information on more cases of biliary cystic tumors because atypical cases were observed, where both OMS and luminal communication with the bile duct were present or absent.**

Keywords: Biliary cystic tumors, ovarian mesenchymal stroma, luminal communication with bile duct

1. Introduction

Biliary cystic tumors, namely biliary cystadenomas or cystadenocarcinomas, form a unilocular or septated multilocular cystic cavity containing mucin. Mural nodules or excrescences may also be observed along the capsular wall (*1-3*). The postulated origin of these lesions is proliferation of the ectopic embryonic tissues that otherwise aid in the development of the adult gallbladder (*4*). The gross and microscopic characteristics of biliary cystadenoma and cystadenocarcinoma distinguish these entities from other hepatic-based cystic lesions, including simple cysts, degenerating metastatic tumors, bilomas, hematomas, abscesses, parasitic diseases, polycystic liver disease, and Caroli disease. Recently, biliary cystic tumors, which are rare hepatic neoplasms, have been divided into 2 clinicopathological groups, based on the presence or absence of ovarian mesenchymal stroma (OMS) and luminal communication with the bile duct. Cystic tumors showing OMS in the cyst wall have been recently classified as mucinous cystic neoplasms (MCNs), according to the World Health Organization (WHO) classification of biliary tumors (*5*). Intraductal papillary neoplasm (IPN) of the bile

**Address correspondence to:*
Dr. Suguru Yamashita, Department of Surgery, Kanto Medical Center NTT EC, Higashi-Gotanda 5-9-22, Shinagawa-ku, Tokyo 141-8625, Japan.
E-mail: origin0304@yahoo.co.jp

duct is the proposed term for describing biliary cystic tumors characterized by the presence of luminal communication with the bile duct. Excessive mucin is frequently produced by the neoplastic cells and the affected bile duct is filled with mucin, showing tubular or cystic luminal dilatation (*6*). This disease, which is distinctly different from MCN, has been recently recognized as IPN of the bile duct according to the WHO classification of biliary neoplasms (*5*).

Each entity resembles MCN and intraductal papillary mucinous neoplasms (IPMNs) of the pancreas, respectively. These entities can be regarded as the biliary counterparts of pancreatic entities (*7-9*). The biologic similarities between the biliary and pancreatic ductal systems have attracted attention because both organs are derived from the ventral endoderm of the foregut (*10*). Similar tumors such as IPNs, intraepithelial neoplasms, and MCNs can arise in biliary and pancreatic ducts (*11-13*).

We have 4 recorded cases of biliary cystic tumors over a 10-year period. In this study, our cases were reclassified and elucidated according to their clinicopathological features. In addition, we performed a search of the Japana Centra Revuo Medicina database and reviewed articles on biliary cystic tumors from 1983 to 2010, with special attention to the presence or absence of OMS and luminal communication of the bile duct. The efficacy and relevance of the subclassifications were analyzed.

2. Case report and review of the literature

2.1. *Report of 4 cases*

Case 1. The patient was a 21-year-old woman. During examination for cholangitis, a multilocular cyst was noted in the right lobe of the liver, and bile duct cystadenoma was suspected based on the Magnetic Resonance Imaging findings. In accordance with the patient's wishes, a wait-and-see approach was adopted; however, the cyst showed a tendency to increase in size, and thus, the possibility of a malignant transformation could not be ruled out. Therefore, an extended resection of the right lobe of the liver was performed. The resected specimen showed a multilocular cyst with a maximum diameter of 11 cm. Further, the cyst contained a viscous, bile-like substance. Histopathological analysis results showed that the cyst was lined with simple cuboidal epithelium showing no atypia, thus establishing the diagnosis of biliary cystadenoma. No ovarian-like stroma was observed. Gallstones were found inside the cyst, suggesting possible communication between the cyst lumen and peripheral bile ducts. Immunostaining results showed that both MUC1 and MUC2 were negative.

Case 2. The patient was a 39-year-old woman. A hepatic cyst was noted as the patient underwent detailed physical and laboratory examination for abdominal pain. The findings showed that the cyst tended to grow in size and that its inner cavity was divided by an emerging septum. Bile duct cystadenoma was suspected, and the left liver lobe was resected. A multilocular cyst with a maximum diameter of 8 cm was found in the resected specimen. Further, a communication between the cyst and bile duct (B4) was macroscopically visible. Histopathological examination results revealed that the cyst was lined with a single layer of cubic columnar epithelium, and an ovarian-like stroma with high cell density was found beneath the epithelium (Figure 1); thus the condition was diagnosed as bile duct cystadenoma. Immunostaining results showed that both MUC1 and MUC2 were negative.

Case 3. The patient was an 80-year-old man. A computed tomography (CT) scan showed the presence of a hepatic cyst during follow-up examination for another disease. Although a wait-and-see approach was adopted, a solid tumor with a tendency to increase in size was detected inside the cyst, and the possibility of bile duct cystadenocarcinoma could not be ruled out. Therefore, a resection of the left lobe was performed. Macroscopic examination of the resected specimen revealed a communication between the cyst and central bile duct. Further, microscopic findings aided in identifying a region showing the transition from a normal bile duct epithelium to an atypical epithelium (Figure 2). No

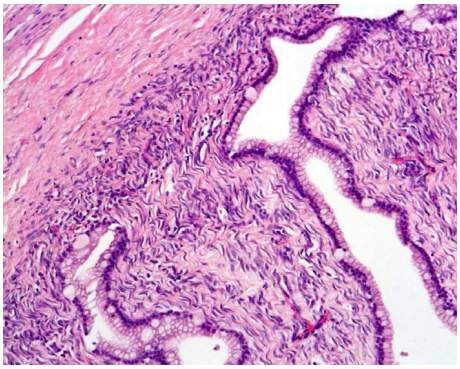

Figure 1. Histological findings revealed the cyst wall consisted of a single layer of cuboidal epithelial cells with ovarian mesenchymal stroma (H & E, ×200).

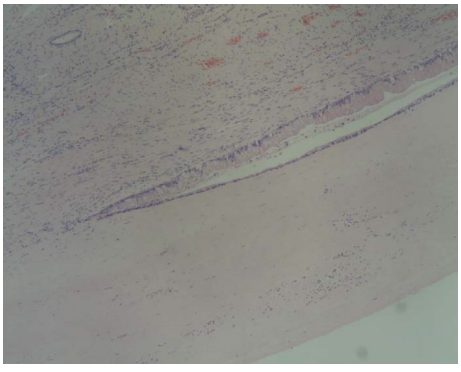

Figure 2. The site of transition from normal biliary epithelium to the atypical epithelium is shown by the histological finding (H & E, ×200).

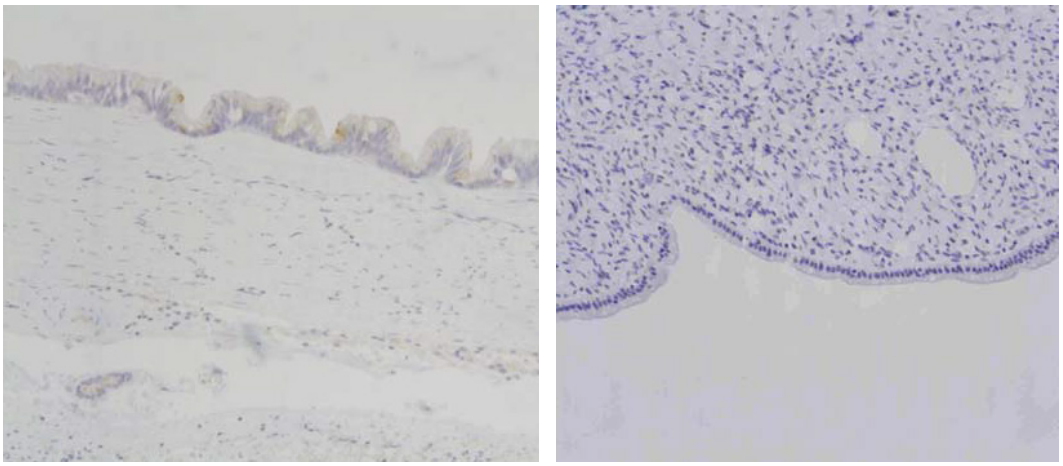

Figure 3. Immunohistochemical examination revealed MUC1+ (left side)/MUC2- (right side) (×40).

ovarian-like stroma and stromal invasion were observed. Thus, histological diagnosis of bile duct cystadenoma was established. However, the finding of an atypical epithelium inside the cyst suggested that unlike the cases of patients 1 and 2, the malignant potential was high in this patient. Immunostaining showed positive results only for MUC1 (Figure 3).

Case 4. The patient was a 61-year-old man. CT scan showed a cystic lesion in the hepatic portal region during follow-up for another disease conducted at our hospital's Department of Internal Medicine. Progressive dilation of the intrahepatic bile ducts was confirmed. Magnetic resonance cholangiopancreatography showed that the dilation started from the B2 bile duct to the left hepatic duct. A solid component was detected inside the B2 bile duct. Mucin-producing cholangiocarcinoma was suspected; therefore, resection of the left lobe of the liver was performed. The resected specimen showed cystic dilation of the intrahepatic bile ducts and contained a tumor with a maximum diameter of 5 cm. Histopathological analysis of the epithelium inside the cyst showed a transition from simple cuboidal epithelium to atypical epithelium with papillary growth (Figure 4). Stromal invasion was observed at the site of the solid tumor, leading to the diagnosis of bile duct cystadenocarcinoma. No ovarian-like stroma was found. Immunostaining results showed that both MUC1 and MUC2 were positive (Figure 5).

Preoperative characteristics, treatment procedures, and histopathological and immunohistochemical findings of the 4 patients are summarized in Table 1. All 4 patients showed a good postoperative course, were discharged after the hospital stay with no complications, and are currently alive with no recurrence.

2.2. *Review of the literature* (Table 2)

In the 117 biliary cystic tumors reported in the Japana Centra Revuo Medicina database between 1983 and 2010, the subtype without OMS and luminal

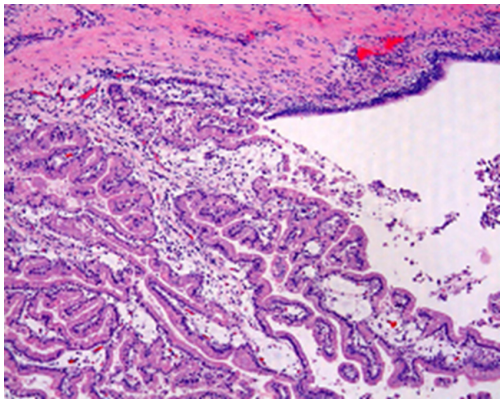

Figure 4. Papillary proliferation of intracystic atypical epithelium and its interstitial infiltration observed (H & E, ×200).

communication with the bile duct occurred at the highest frequency [39.3% (n = 46)]. On the other hand, only 4 tumors (3.4%) were found to have both OMS and luminal communication with the bile duct. Among 43 tumors with luminal communication with the bile duct, 35 tumors (81.4%) showed communication between the main biliary duct and the cystic tumor. The main biliary duct was defined as the primary and secondary branches of the biliary tree. As for gender distribution, the tumors with OMS occurred in all 32 women. Although adenocarcinoma was seen in 2 out of 28 tumors (7.1%) that showed OMS rather than luminal communication with the bile duct, as many as 28 of the 39 patients (71.8%) with luminal communication with the bile duct rather than OMS had adenocarcinoma.

3. Discussion

First, it seems reasonable to divide biliary cystic tumors that originally included biliary cystadenoma and biliary cystadenocarcinoma into IPN of the bile duct and hepatic MCN, as is the case for the pancreas. The existence of OMS contributed to the subclassification

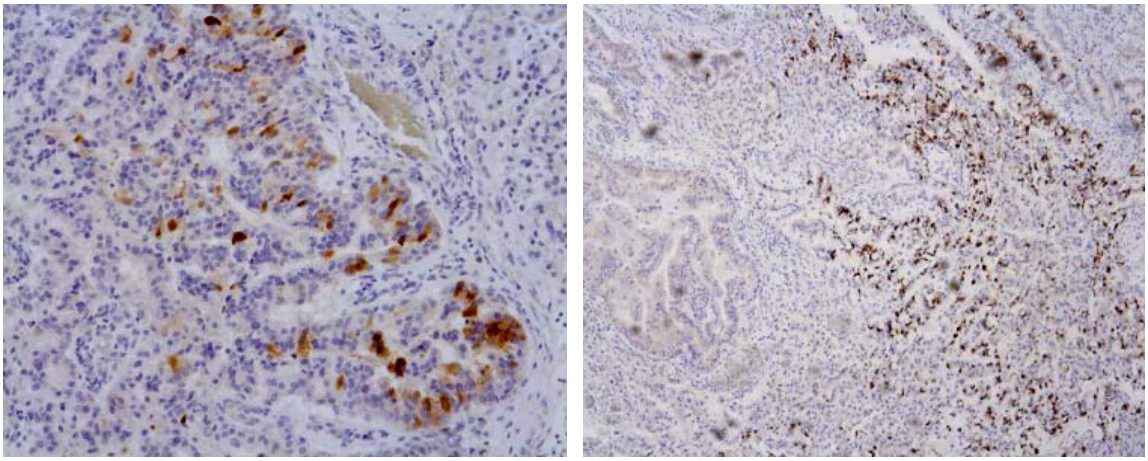

Figure 5. Immunohistochemical examination reveals MUC1+ (left side)/MUC2+ (right side) (×100 and ×40, respectively).

Table 1. Characteristics of our cases

Items	Case 1	Case 2	Case 3	Case 4
Gender	Female	Female	Male	Male
Age(y)	21	39	80	61
Clinical Findings	None	Abdominal pain	None	None
Clinical Diagnosis	Biliary cystadenoma	Biliary cystadenoma	Biliary cystadenoma	Mucus-producing cholangio-carcinoma
Operative Procedure	Extended right hepatectomy	Left hepatectomy	Left hepatectomy	Left hepatectomy
OMS	Absence	Presence	Absence	Absence
Luminal communication with bile duct	Presence	Presence	Presence	Presence
Atypicality of intracystic epithelium	Absence	Absence	Presence	Presence
Interstitial infiltration	Absence	Absence	Absence	Presence
Expression of mucin protein	MUC1-/MUC2-	MUC1-/MUC2-	MUC1+/MUC2-	MUC1+/MUC2+
Traditional histopathological diagnosis	Biliary cystadenoma	Biliary cystadenoma	Biliary cystadenoma	Biliary cystadenocarcinoma
New histopathological diagnosis	IPN of the bile duct	MCN and IPN of the bile duct	IPN of the bile duct	IPN of the bile duct

OMS, ovarian mesenchymal stroma; MUC, mucin; IPN, intraductal papillary neoplasm; MCN, mucinous cystic neoplasm.

Table 2. Characteristics of the reported cases in our review

(a)

Items	OMS (-)	OMS (+)	Total
Luminal communication with bile duct (-)	46 (39.3)	28 (23.9)	74 (63.2)
Luminal communication with bile duct (+) (main duct type)	33 (28.2)	2 (1.7)	35 (29.9)
Luminal communication with bile duct (+) (branch duct type)	6 (5.1)	2 (1.7)	8 (6.8)
Total	85 (72.7)	32 (27.4)	117 (100)

(b)

Items	OMS (-)	OMS (+)
Luminal communication with bile duct (-)		
Female	31/46 (67.4)	28/28 (100)
Male	15/46 (32.6)	0/28 (0)
Adenocarcinoma	29/46 (63.0)	2/28 (7.1)
Adenoma	17/46 (37.0)	26/28 (92.9)
Luminal communication with bile duct (+)		
Female	21/39 (53.8)	4/4 (100)
Male	18/39 (46.2)	0/4 (0)
Adenocarcinoma	28/39 (71.8)	1/4 (25)
Adenoma	11/39 (28.2)	3/4 (75)

Values in parentheses are percentages. OMS, ovarian mesenchymal stroma. The main biliary duct was defined as the primary and secondary branches of the biliary tree.

of biliary cystic tumors in epithelial tumors of the liver, analogous to IPMN and MCN in pancreatic epithelial tumors, according to the WHO classification. As seen in case 4 from our institution, a mucus-producing cholangiocarcinoma or an intra-bile duct tumor growth type of intrahepatic cholangiocarcinoma, which showed a poor tendency for interstitial infiltration, might be involved in IPN of the bile duct. In contrast to cases 1, 3, and 4, which were considered typical IPN of the bile duct because they lacked OMS but showed luminal communication with the bile duct, case 2 was an atypical form because it showed both luminal communication with the main biliary duct and OMS. Occasionally, we have seen atypical forms of pancreatic mucin-producing pancreatic neoplasms, including IPMN and MCN (*14*).

Second, the presence or absence of MUC1 and MUC2 expression could be an indicator of the malignant grade of the tumor. During the past several years, a number of human mucins (MUC1-MUC9) have been identified (*15-23*). MUC1 is a membrane-associated glycoprotein with an extracellular domain consisting of a variable number of highly conserved tandem repeats of 20 amino acids, a transmembrane domain, and a cytoplasmic tail of 69 amino acids (*24,25*). MUC2-MUC7 are expressed by secretory cell types (*26,27*), whereas MUC8 and MUC9 are expressed in the reproductive tract tissues (*22,23*). MUC2 expression has been frequently observed in biliary papillary tumors, including non-invasive tumors and invasive lesions (tubular adenocarcinoma and mucinous carcinoma), whereas MUC1 expression is commonly found in tubular adenocarcinoma cases but rarely in non-invasive tumors and mucinous carcinoma (*28*). Pancreatic IPMN is commonly divided into 4 histopathological subtypes: pancreatobiliary, intestinal, gastric, and oncocytic subtypes. These subtypes correlate well with tumor cellular atypism (*28*). The pancreatobiliary, intestinal, and oncocytic subtypes tend to occur in the main pancreatic duct-type IPMN and are frequently associated with invasive carcinoma. However, the gastric type tends to occur in branched-type IPMN and appears to be benign (*29*). Furthermore, these subtypes show differences in MUC expression. The pancreatobiliary, intestinal, oncocytic, and gastric subtypes are often MUC1+/MUC2±, MUC1-/MUC2+, MUC1+/MUC2±, and MUC1-/MUC2-, respectively. Thus, either MUC1+ or MUC2+ seems to be associated with an increased risk of malignancy. In the above mentioned cases, case 3 showed atypical epithelial cells and was MUC1+/MUC2-, and case 4, which included papillary proliferation of atypical epithelium and interstitial infiltration, was MUC1+/MUC2+. In contrast, cases 1 and 2, which had normal intracystic simple cuboidal epithelium, were MUC1-/MUC2-. Furthermore, Zen *et al*. revealed that IPN of the bile duct commonly was MUC1+/MUC2±, whereas pancreatic IPMN was often MUC1-/MUC2± (*30*). Therefore, although IPN of the bile duct and pancreatic IPMN are thought to be related diseases, the former should be given more attention because it might have a higher malignant potential. MUC immunohistochemistry, which is the prevalent technique in pancreatic IPMN, could provide a good indication of the malignancy grade of IPN in the bile duct.

Interestingly, our analysis of these past cases revealed that biliary cystic tumors with OMS all occurred in women as equally as the findings in pancreatic IPMN. Similarly, the cases without OMS had a higher tendency to be adenocarcinoma than cases with OMS. Thus, clinicopathological subclassification of biliary cystic tumors, according to the presence or absence of OMS and luminal communication with the bile duct, is required for further recognition of the pathophysiology and prognosis. However, since several intermediate or transitional types have been detected, with the exception of the typical type, further accumulation and elucidation of clinical cases are required.

Since many cases with malignant potential or malignancy itself were observed, such as our case series, radical hepatic resection is the recommended treatment for IPN of the bile duct and hepatic MCN as long as the patient is operable. All 4 of our patients survived and showed no recurrence.

In conclusion, research on more cases of biliary cystic tumors must be conducted and it is reasonable and appropriate to subclassify biliary cystic tumors according to the presence or absence of OMS and luminal communication with the bile duct. Hepatic resection is recommended as the curative treatment for biliary cystic tumors because they reveal various grades of malignancy.

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Commentary

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Necessity of cooperation with government on publication of scientific research results for intractable diseases

Yoshinori Inagaki[1,2,*], Peipei Song[2]

[1] *Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan;*
[2] *Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.*

Summary **The features of intractable diseases make it an important public health issue and a challenge to medical care worldwide. Investigation of intractable diseases with the support of government is urgently expected to activate clinical and pharmaceutical research to promote diagnosis and treatment for patients with intractable diseases. Moreover, linkage to the international database for research achievement is also necessary so that both researchers and other general citizens can assess research trends in the field of intractable diseases. In Japan, supportive activities for patients and researchers of intractable diseases have been well developed with the support of the Ministry of Health, Labor and Welfare (MHLW). Furthermore, in April 2013, a specific academic communication platform on intractable diseases – the Intractable and Rare Diseases Research (IRDR) Journal – was approved to join a governmental project and receive support from the Japan Society for the Promotion of Science (JSPS) under the auspices of Ministry of Education, Culture, Sports, Science and Technology (MEXT). Cooperation with the Japanese government starting this year is hoped to promote information-sharing based on an academic communication platform and further activate research on intractable diseases.**

Keywords: Intractable diseases, rare diseases, orphan drugs, governmental supports

There are many patients suffering from intractable diseases worldwide which have an uncertain etiology, no effective therapeutic methods, and heavy burdens on not only patients but also other family members financially and mentally. Several countries such as the United States, European and some Asian countries deploy various efforts for the purpose of medical and financial rescue for those patients (*1*). Most intractable diseases are chronic diseases, therefore continuous financial support from government is essential for patients. On the other hand, financial supports have also been provided to investigations regarding intractable diseases. Those supports contribute to activate clinical and pharmaceutical research to survey the patients' prognosis and develop effective orphan drugs (*2*). Newly-developed technologies are actively applied. Development of current life science technology such as genomic analysis using next-generation sequencing and regenerative medicine using induced pluripotent stem (iPS) cells contributes to the understanding of pathogenetic mechanisms and establishment of new diagnostic or therapeutic strategies (*3,4*). However, progression of this research takes a fair amount of time because each disease has characteristic pathogenic mechanisms and some of the diseases, called "rare diseases", have a fewer number of patients. Thus, a long-term continuous effort of support for research is also necessary for academic research.

Supportive activities for patients and researchers of intractable diseases are also deployed in Japan. The Ministry of Health, Labor and Welfare (MHLW) mainly administers those activities; epidemiological investigation regarding the current state of intractable diseases in Japan, provision of proper medical care and its financial support for patients, administration

**Address correspondence to:*
Dr. Yoshinori Inagaki, Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: yinagaki-tky@umin.ac.jp

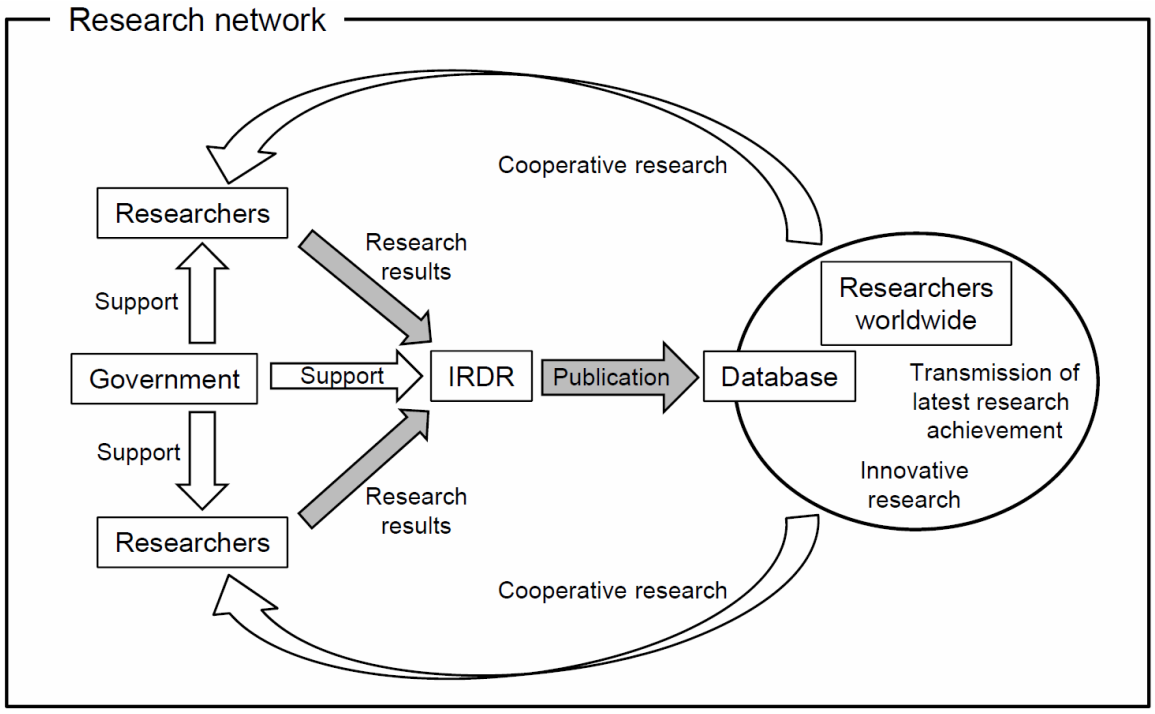

Figure 1. Government-supported academic communication platform for publication of scientific research results for intractable diseases. IRDR receives financial support from Japanese government and contributes to create a cooperative research network in the field of intractable diseases.

and supports of innovative academic research. Japan Intractable Diseases Information Center (JIDIC) was established by the cooperative enterprise of MHLW and the Japan Intractable Diseases Research Foundation for the purpose of disclosure of information about intractable diseases mainly in Japan. The website of JIDIC provides information on *i*) intractable diseases (130 diseases which MHLW approved as "Nanbyo"), *ii*) domestic researchers studying intractable diseases, and *iii*) medical welfare system for patients with intractable diseases. The number of accesses to this website is currently over 15 million per year, and therefore this website becomes significant to understand the present state of each intractable disease in Japan. However the description of the latest research achievements in foreign countries is inadequate on this website. Linkage to the international database for research achievements is required so that both researchers and other general citizens can assess the research trends in the field of intractable diseases. Information-sharing and implementation of cooperative work among domestic and foreign researchers are considered necessary to overcome each intractable disease as early as possible.

International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) group promotes cooperation in the field of intractable and rare diseases research *via* publication of the present journal "Intractable and Rare Diseases Research (IRDR)". This journal consolidates significant research achievements regarding intractable and rare diseases scattered in various academic fields and introduces the latest results aggressively. In addition, a database summarizing individual proposed research plans is being constructed to activate intercommunication between researchers. This project leads information-sharing and implementation of cooperative work among researchers. Activation of the research field of intractable and rare diseases is expected to contribute to overcoming those diseases. Last April, the present journal, IRDR, was approved to join a governmental project and receive support from Japan Society for the Promotion of Science (JSPS) under the auspices of Ministry of Education, Culture, Sports, Science and Technology (MEXT). The purpose of this project, named "Grant-in-Aid for Publication of Scientific Research Results", is to construct a system that can transmit the limited research results to an international network. In Japan, until now, there has been no project constructing an international system regarding consolidation and transmission of research achievements regarding intractable and rare diseases with a financial support from government; furthermore, there is no journal like IRDR that focuses on the topics of intractable and rare diseases. In this journal, novel and significant research results are actively published and rapidly transmitted worldwide *via* internationally influential databases. The governmental support is essential to execute those plans (Figure 1). Cooperation with government that started this year is hoped to elevate this journal to an internationally high-impact one and activate the research field of intractable and rare diseases.

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IRDR
Intractable & Rare Diseases Research

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Pearl City Koishikawa 603
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