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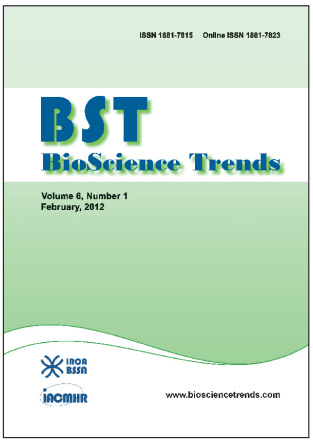

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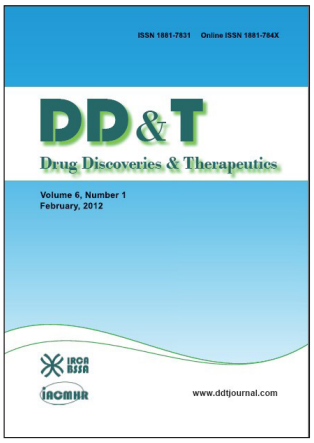

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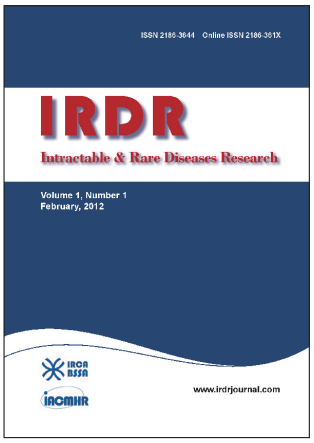

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Editorial and Head Office

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
URL: www.irdrjournal.com

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Review

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The current status of orphan drug development in Europe and the US

Anthony K Hall[1,*], Marilyn R Carlson[2]

[1] *PSR Orphan Experts, Hoofddorp, Netherlands and Findacure Development, Cambridge, UK;*
[2] *Agility Clinical, Carlsbad, CA, USA.*

Summary **Orphan drug legislation has been introduced in a number of countries in order to stimulate the development of treatments for rare diseases by introducing commercial incentives for companies wishing to undertake that development. In order to navigate the maze of regulatory regulations and procedures so that companies can make proper use of the orphan drug incentives, specialist knowledge is required. This article will review the current status of orphan drug development in the EU and the US, explain the incentives and procedures, and touch on the role of patient organisations in the process.**

Keywords: Rare diseases, orphan drugs, orphan designation, patient organisations

1. Introduction

"Patients suffering from rare conditions should be entitled to the same quality of treatment as other patients". This laudable sentiment is enshrined in European law in the landmark European regulation 141/2000 (*1*) which introduced a set of commercial incentives in European Union (EU) to try to stimulate the development of products for rare (orphan) diseases, which until then had been largely neglected by the pharmaceutical industry.

In the US, there are more than 25 million people affected by more than 7,000 diseases that are considered rare. With only 10 new drugs approved for rare diseases between 1972 and 1983, parents and caregivers enlisted the support of legislators and the Orphan Drug Act (ODA) became law in 1983 (*2*). The US was the first nation to introduce orphan drug legislation and, in the intervening years, a number of other countries have followed suit, for example Japan (1993), Singapore (1997), Australia (1998) and the EU (2000). Other countries, such as Canada, recognize the importance of orphan drug legislation and consider applications for rare diseases on a case-by-case basis, but have yet to issue regulations.

Whilst there is no doubt that the incentives have been of great benefit, the cause of the recent explosion of interest in orphan drug development is likely to be multifactorial, *e.g.* patent expiration for blockbuster drugs; the lack of innovative treatments for well-established diseases; the growth of the biotechnology industry; the advances in molecular biology; the considerable growth in the effectiveness of rare disease patient groups; and the ubiquitous availability of social media.

2. Incentives for orphan drug development

The main incentives of the orphan drug legislation in the EU and the US are shown in Table 1. The market exclusivity is usually considered to be the most important of the incentives, although this only comes into effect after marketing authorisation (MA) has been granted in the EU and after the new drug application (NDA) has been approved by the Food and Drug Administration (FDA) in the US.

In the EU, the market exclusivity prevents another application for a MA (and also the extension of an existing MA) for the same therapeutic indication, for a similar medicinal product. It is possible (and indeed quite common) for multiple products to obtain orphan designation for the same indication but, for two similar

**Address correspondence to:*
Dr. Anthony Hall, Co-founder, PSR Orphan Experts, Planetenweg 5, 2132HN Hoofddorp, Netherlands.
E-mail: tony.hall@psr-group.com
Co-founder, Findacure Development, 66 Devonshire Road, Cambridge CB1 2BL, UK.
E-mail: tony@findacure.org.uk

products, only the first to obtain MA will be granted the MA and the 10-year exclusivity. For example, in the EU, 16 products have received orphan designation for pulmonary arterial hypertension, but only 4 have received MA. A second product may be granted a MA for the same indication, if it is not similar to the first product, although it would still be necessary to demonstrate significant benefit if it was to obtain orphan designation. Exceptions to these rules exist if the original MA holder gives consent, or the original MA holder cannot supply sufficient product or the second applicant can show that their product is safer, more effective or otherwise clinically superior (*5*).

In the US, orphan drug designation is conferred to the active moiety in the product and, as in the EU, the first active moiety with orphan designation to reach the market receives the benefits of exclusivity. If a product has received marketing approval in the US for use in an orphan indication, the only way another product can be designated as an orphan drug for that indication is if the sponsor provides a reasonable hypothesis that their product is "clinically superior" to the approved product by means of greater effectiveness, greater safety, or that it provides a major contribution to patient care. Any claim for clinical superiority could require a head-to-head trial (*6*).

During the development programme, the free advice and fee reductions from the regulatory agencies can also be extremely helpful incentives. In the EU, these incentives generally favour small and medium-sized enterprises (SMEs), but are revised from time to time as shown in Table 2.

In the US, orphan drug designation gives sponsors an exemption from the fees required when filing a new drug or biologic application. For fiscal year 2014, the fee for an application requiring clinical data is $2,169,100 under the Prescription Drug User Fee Act (PDUFA).

The ODA is widely recognised as having been successful in encouraging the development of products for orphan indications in the US and the EU. Since it was enacted in 1983, there have been nearly 3,000 orphan designations and 448 approvals in the US. In 2008, more than a third of all FDA-approved new chemical entities (NCEs) were orphan drugs. In the EU, there have been 1,219 products designated and 78 approved (excluding those withdrawn and expired, there are currently 986 designated and 67 approved orphan drugs) (*7*).

Table 1. Key incentives of the orphan drug legislation in Europe and the US*

Items	EU	US
Market exclusivity	10 years[a,b]	7 years[d]
Protocol assistance and follow-up	Yes	Yes
Reduced / waived regulatory fees	Yes	Yes
Tax credit on clinical trials	No	Yes
Specific subsidies for clinical trials	No[c]	Yes

[a] plus an extra 2 years if paediatric development included; [b] may be reduced to 6 years if the product is sufficiently profitable; [c] EU funding is available for rare diseases in addition to various national grants; [d] plus an additional 6 months of paediatric exclusivity for qualified studies. *Adapted from reference (*3*) and (*4*).

3. Obtaining orphan designation

The incentives outlined above apply to products which have obtained designation as an orphan drug (orphan medicinal product in the EU). An orphan designation application may be submitted at any time in development, but before the submission of the application for marketing authorisation. In the US, the application for orphan designation is assessed by the Office of Orphan Products Development (OOPD), a branch of the FDA; in the EU it is assessed by the Committee for Orphan Medicinal Products (COMP), one of the committees of the EMA.

For a drug to qualify for orphan designation in the US, both the drug and the disease or condition must meet criteria specified in the US Code of Federal Regulations (CFR) Title 21 Part 316 Orphan Drugs (*2*). The regulations define "rare disease or condition" as any disease or condition which affects less than 200,000 individuals in the US, or if it affects more than 200,000 persons in the US, there is "no reasonable expectation"

Table 2. Recent changes in fee reductions in Europe for companies developing orphan medicinal products

Items	2013	2014
Protocol assistance and follow-up	100 for SMEs 40% for non-SMEs (non-paediatric-related assistance) 100% for non-SMEs (paediatric-related assistance)	100 for SMEs 75% for non-SMEs (non-paediatric-related assistance) 100% for non-SMEs (paediatric-related assistance)
Pre-authorisation inspections	100% for SMEs	100%
Initial marketing authorisation application	100% for SMEs	100% for SMEs 10% for non-SMEs
Post-authorisation activities, including annual fee in the 1st year after MA	100% for SMEs	100% for SMEs

that the cost of developing and making the drug available in the US for this rare disease or condition will be recovered from US sales of the drug.

The prevalence of the disease for which the treatment is being developed must be less than 200,000 persons in the US who have been diagnosed at the time of the submission of the request for orphan drug designation. For a vaccine or a drug to prevent a rare disease or condition, the estimated number of people to whom the drug will be administered annually must be included with a basis for the estimate. When there is no reasonable expectation that the cost of research and development can be recovered by sales of the product in the US, the sponsor must submit detailed documentation of the development costs incurred and the anticipated market for the drug.

In the EU, the prevalence must be below 5 per 10,000 of the EU population, except (in a comparable way to the US law) where the expected return on investment is insufficient to justify the investment.

There has been a certain degree of harmonisation of the procedures for designation on either side of the Atlantic (*e.g*. the introduction of a common application form) but there remain some differences between the procedures, as outlined in Table 3.

One of the key differences between the orphan designation process in the EU and US is that, in the latter the emphasis is on demonstrating the scientific rationale and disease prevalence, whilst in the EU there are two additional requirements: *i*) that the condition is life-threatening or seriously debilitating, and *ii*) that there is currently either no satisfactory method (of diagnosis, prevention or treatment) or that the new product will be of significant benefit over the existing method. Any product with a MA in any EU country would be considered to be a "satisfactory" method and, even where a product is not authorised, if it is widely used it may be considered to be satisfactory. In such a case, the applicant needs to provide a solid argument as to why the new method is expected to be superior.

The most challenging part of the application is usually obtaining reliable prevalence data. For the EU, the criteria require demonstrating prevalence *in the European Community* and it is not enough to cite prevalence figures for one or two countries only. For many rare diseases, there may be very little information available in the literature on the epidemiology of the disease. It is not considered adequate to state that the prevalence "obviously" meets the criterion, nor to simply quote sources such as OrphaNet. Instead, it is necessary to provide a properly referenced analysis and, if the prevalence figure is close to the cut-off of 5 per 10,000, some sensitivity analyses may also be needed to convince the COMP that the true prevalence is really within the limits.

The process of orphan designation can take up to six months in the EU. The sponsor needs to submit a Letter of Intent at least two months before the intended submission date, which allows time for the COMP to appoint the coordinators at one of its regular monthly meetings. Subsequently, a pre-submission meeting can be very helpful to informally discuss the draft application and obtain feedback from the coordinators on likely weaknesses in the application, so that these can be addressed before submission. After submission, there is a process of validation, during which the content of the application is examined for completeness and for conformity to the guidelines. Only after this

Table 3. Key differences in the procedures for orphan designation in the EU and US*

Items	EU	US
Terminology	Orphan medicinal product designation	Orphan drug designation
Application to	Committee for Orphan Medicinal Products	Office of Orphan Products Development
Timetable	Timetable for submission and assessment published by EMA	Any time; no defined timetable
Prevalence criteria	Disease or condition affects < 5 in 10,000 persons in the EU	Disease or condition affects < 200,000 persons in the US
Dossier	Sections A-E according to ENTR/6283/00	Nine parts according to 21 CFR 316.20
Key aspects of the application	Medical plausibility Prevalence Justification of significant benefit or why other methods are not satisfactory	Scientific rationale Prevalence
Sponsor established in territory	Proof of establishment in EU	Not required
Translations	Translations of product name and proposed orphan indication into all official languages of the EU plus Icelandic and Norwegian	Not required

*Adapted from references of (*2,4,8-10*).

does the clock start. Sixty days after the clock start, the COMP considers the application at its meeting and the application can receive a positive opinion at this stage. Quite commonly however, the COMP has some questions, which are communicated to the applicant as a List of Issues. The sponsor then has the opportunity to address these in writing and in person at an oral explanation, which occurs 90 days after the clock start at the next COMP meeting. The COMP is required to issue an opinion at this point. A positive opinion is sent to the European Commission (EC) for ratification, which is supposed to occur after a further 30 days. When a negative opinion from the COMP is inevitable, the applicant is given the opportunity to withdraw the application at that stage, in order to avoid the publication of a negative opinion.

In the US, the process is less complicated. A request for orphan designation is submitted in writing to the FDA OOPD. The sponsor may request orphan designation of a previously unapproved drug, or for a new use of an already marketed drug. In addition, a sponsor of a drug that is otherwise the same drug as an already approved drug may seek and obtain orphan drug designation for the subsequent drug for the same rare disease or condition if it can present a plausible hypothesis that its drug may be clinically superior to the first drug. More than one sponsor may receive orphan-drug designation for the same drug for the same rare disease or condition, but each sponsor seeking orphan-drug designation must file a complete request for designation. The application must include prevalence data and a scientific rationale that establishes a medically plausible basis to expect the drug will be effective for the rare disease. Clinical trial data is preferred but, in the absence of data in humans, "compelling" pre-clinical data in a relevant animal model may be adequate to support the scientific rationale. Animal toxicology data that describes the safety of the drug is not acceptable support for the scientific rationale.

For a drug used to treat a rare disease or condition, the application requires documentation with authoritative references of the prevalence of the disease or condition. For products to be used for a condition of less than one year in duration, incidence may be used as an estimate of the target population. For a product to be used as a preventative (*e.g.* a vaccine), the application requires documentation of the number of people to whom the drug will be administered annually. If the basis for the application is that there is no reasonable expectation of recovering the costs of development, justification must be provided for production and marketing costs the sponsor has incurred and expects to incur during the first seven years after the drug is marketed in the US (*2*).

To apply for designation in the US, the sponsor can follow the content and format described in the regulations (21 CFR 316.20) and use the common application (Form FDA 3671). The common application form includes items that are specific to the EU, however, if a sponsor is going to apply for orphan drug designation in both the EU and US, the common application form may be useful (*11*).

Once received by the OOPD, the application is assigned a designation application number, entered into the OOPD database, and an acknowledgement letter is sent to the sponsor. A reviewer is assigned and the reviewer prepares a review which is sent to the OOPD Team Leader for a second level review and concurrence. The review is forwarded for a third level review by the OOPD Office Director. Following the OOPD Director's concurrence, a designation letter, a letter requesting additional information, or a denial letter is prepared for the Director's signature and the letter is issued to the sponsor. The information is not made public unless the orphan designation is granted. On the FDA website there is a searchable database listing all orphan designations and approvals (*12*).

Every foreign sponsor that plans to apply for orphan drug designation is required to have a US agent to correspond with the FDA on their behalf. The agent must be a permanent resident and may be an individual, a firm, or a domestic corporation and may represent any number of sponsors. The name of the permanent-resident agent, address, telephone number, and email address must be submitted to the OOPD (*13*).

4. After orphan designation

Companies sometimes fix their objectives on obtaining orphan designation, as if this is the end of the process, when it is usually just the beginning. This may be because, for some small companies, it is an important milestone which can be used to attract investors. However, it is important to realise that the development of an orphan drug needs to be performed in the proper way to ultimately lead to a positive benefit-risk assessment by the regulatory agencies.

Some companies make the mistake of thinking that "orphan status" will automatically allow them to obtain an expedited MA/NDA based on a single small study. Although there are now many examples of orphan drugs which have been granted approval based on very small clinical development programmes, this is often due to the fact that the treatment effect can be quite large in these serious, often fatal diseases, for which there is no other therapeutic option. In the end, the regulators still need to be convinced that the product is safe and effective. This is one of the reasons why it is very useful to obtain protocol assistance (the equivalence of scientific advice for orphan drugs) from the European Medicines Agency (EMA) prior to embarking on a non-conventional development programme.

Similarly, in the US, the granting of an orphan designation request does not alter the standard regulatory requirements and process for obtaining

marketing approval. Safety and effectiveness of a drug must be established through adequate and well-controlled studies (*14*).

5. Special regulatory procedures

Notwithstanding the above commentary, there are some special regulatory procedures which, although not specific for orphan drugs, are more likely to apply to products for rare diseases. Some of these procedures are common to both the EU and US, whilst others are specific for each territory, as shown in Table 4.

It is noteworthy that, of the 67 currently approved orphan medicinal products in the EU, 4 have conditional approval and 14 have MA under exceptional circumstances.

In 2012, FDA signed into law the Food and Drug Administration Safety and Innovation Act (FDASIA) which created a new expedited drug development tool, known as the "breakthrough therapy" designation (*18*). This new designation allows FDA to assist drug developers to expedite the development and review of new drugs that have preliminary clinical evidence that indicates the drug may offer a substantial improvement over available therapies for patients with serious or life-threatening diseases.

In 2013, FDA issued a draft guidance that provides detailed information about breakthrough designation and other expedited approval programs; *Expedited Programs for Serious Conditions — Drugs and Biologics* June 2013 (*19*).

As shown in Table 5, the four programs intended to expedite development and review of drugs to address unmet need in the treatment of serious or life threatening conditions are fast track designation, breakthrough therapy designation, the accelerated approval pathway and priority review.

6. National Plans for rare diseases

Moving away from the purely regulatory aspects of orphan drugs, it is useful to have a look at the framework

Table 4. Comparison of regulatory procedures in the US and EU intended to accelerate the approval of drugs which fulfil an unmet medical need*

FDA	EMA
Fast Track Designation Rolling review to help expedite the process. More meetings with FDA.	No equivalent
Breakthrough Therapy Designation Intensive guidance on efficient drug development. Organizational commitment involving senior managers.	No equivalent
Accelerated Approval Pathway Approval with a surrogate or intermediate clinical endpoint. Approval is conditional on post-approval trials showing clinical benefit, after which FDA grants a traditional approval.	*Conditional Approval* Incomplete data; not specific for surrogate endpoint. Approval is conditional on providing additional post-approval data. After confirmation, authorisation is converted to a normal approval.
No equivalent	*Approval under exceptional circumstances* Incomplete data. It is not expected that compete data can ever be provided.
Priority Review Designation Reduced time for review of NDA Reduced from 10 to 6 months	*Accelerated Assessment* Reduced time for review of MAA Reduced from 210 to 150 days

*Adapted from references of (*15-17*).

Table 5. FDA Expedited Programs for Serious Conditions

Items	Fast track designation	Breakthrough therapy designation	Accelerated approval pathway	Priority review designation
When to submit	With IND and no later than the pre-BLA or pre-NDA meeting	With IND and no later than the end-of-Phase 2 meeting	Discuss with FDA review division	With original BLA, NDA, or efficacy supplement
FDA response	Within 60 calendar days	Within 60 calendar days	Not specified	Within 60 calendar days
Reference	FDA Modernization Act of 1997 (FDAMA), amended by FDASIA	Section 506 (a) of the FD&C Act, as added by FDASIA	21 CFR part 314, subpart H; 21 CFR part 601, subpart E; Section 506(c) of the FD&C Act, as amended by FDASIA	Prescription Drug User Fee Act of 1992

within which the orphan drugs expansion is occurring. There is no doubt that the rare diseases patient groups have been instrumental in the creation, first of the orphan drugs legislation and later of the introduction and expansion of rare diseases services within Europe. EURORDIS and NORD, the umbrella organisations for rare diseases patient groups in Europe and the US, have been key players in lobbying for change.

One of the important consequences of the push for change was the publication of a European Commission recommendation that all EU countries should "elaborate and adopt a (national) plan or strategy as soon as possible, preferably by the end of 2013 at the latest, aimed at guiding and structuring relevant actions in the field of rare diseases within the framework of their health and social systems" (*20*). The recommendation also proposed the setting up of Centres of Expertise and European Reference Networks for rare diseases. The enthusiasm with which the recommendation has been taken up by individual Member States has been variable with, for example, France publishing its first National Plan for rare diseases in 2004, whilst the UK eventually published its first plan only in late 2013.

In addition to the umbrella groups, individual patient groups have also been very productive at a "grass roots" level, organising the setting up of National Centres, raising money to fund basic research and, in some cases, even driving the clinical development of treatments by establishing consortia of stakeholders which can together undertake large projects previously only possible by pharmaceutical companies (*21*).

7. Pricing and value of orphan drugs

It is inevitable that, when the total market for a new treatment is very small, as is the case for orphan drugs, the price that the manufacturer will charge is likely to be significantly higher than for drugs to treat common diseases. In many cases, the cost is several hundred thousand dollars/Euros per patient per year. With ever-increasing pressures on health budgets, the reimbursement bodies have started to look very critically at the cost-benefit ratio for orphan drugs, leading to a situation where many orphan drugs, although approved throughout Europe, are not available in some countries due to lack of funding.

In the US, patient access to expensive orphan drugs is limited by the patient's own lack of health insurance or their inability to pay the portion of the cost of their treatment not covered by their insurance plan.

Various approaches have been proposed to tackle the issue of "value" for orphan drugs, see for example Hughes-Wilson *et al*. (*22*), but to date none has been adopted. The prospect that, in the future, orphan drugs might find it increasingly difficult to get reimbursed is a potentially negative consequence of an otherwise very positive move to provide treatments for patients with rare diseases.

6. Conclusion

The orphan drug legislation in the US and the EU has been successful in enabling patients with rare diseases to receive treatments that would otherwise never have been developed. More than 30 years after the ODA was approved in the US and 14 years since the European Regulation, this has led to the approval of 448 orphan products in the US and 78 in Europe. In addition to the incentives within the original legislation, there are now additional regulatory pathways which help to expedite the development and approval of drugs for conditions of unmet medical need. In addition, the deeper involvement of patient organisations in the drug development process itself, rather than purely as lobbyists, is changing the way that orphan drugs are developed.

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Mini-Review

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Human Mendelian diseases related to abnormalities of the RNA exosome or its cofactors

Alexandre Fabre[1,2,*], Catherine Badens[2,3]

[1] *Service de pédiatrie Multidisciplinaire, Hôpital des Enfants de la Timone, APHM, Marseille, France;*
[2] *UMR_S 910, Aix-Marseille Université, Marseille, France;*
[3] *Service de Génétique Moléculaire, Hôpital des Enfants de la Timone, APHM, Marseille, France.*

Summary **The RNA exosome has a key role in RNA decays and RNA quality control. In 2012, two human Mendelian diseases: syndromic diarrhea/tricho-hepato-enteric syndrome (SD/THE) and Ponto-cerebellar hypoplasia type 1(PCH1) were linked to the RNA exosome or its cofactor's defect. SD/THE's main features are an intractable diarrhea of infancy associated with hair abnormalities, facial dysmorphism, intra uterine growth restriction and immune deficiency. SD/THE is caused by a defect of the SKI complex (*TTC37* and *SKIV2L*), the cytoplasmic co-factor of the RNA exosome for mRNA degradation. PCH1's main features are atrophy of the pons and of the cerebellum, a progressive microcephaly with developmental delay and muscle atrophy secondary to spinal anterior horn cell loss. In 30-40% of patients, PCH1 is caused by a defect in *EXOSC3* which encodes RRP40, a protein of the cap of the RNA exosome. Thanks to knowledge about other forms of PCH it could be assumed that the altered substrates are probably transfer RNA However, as there exists no patient with two null mutations, residual RNA exosome functionality is probably required to preserve viability. Thus, to date two very different human Mendelian diseases have been related to the dysfunctioning of the RNA exosome. It illustrates the versatility of the RNA exosome function and substrate.**

Keywords: Syndromic diarrhea, tricho-hepato-enteric syndrome, RNA exosome, Ponto-cerebellar hypoplasia type 1, *EXOSC3*, *TTC37*, *SKIV2L*

1. Introduction

In 2012, two human Mendelian diseases were linked to the RNA exosome or its cofactor. The RNA exosome, discovered in 1997, has a key role in the RNA decays and RNA quality control (*1,2*). Mostly studied in yeast, it is conserved among eukaryotes and there exist homologs in archea. In yeast, the RNA exosome is a multiprotein complex (Figure 1). The core of the RNA exosome consists of 9 proteins which form a barrel-like structure. Six proteins form the ring of the exosome (Rrp41, Rrp42, Rrp43, Rrp45, Rrp46, Mtr3) on top of which stands the cap of exosome, formed by three proteins (Csl4, Rrp40, Rrp4). Most of the enzymatic activity is brought by associated proteins RRP44 and RRP6 which also have other functions and subcellular localization. Finally two cofactors are needed for RNA exosome function: Mtr4 either alone or with the TRAMP complex (Air1/2, Trf4/5 and Mtr4) and the SKI complex (Ski2, Ski3 and Ski8). The RNA substrates are varied and include messenger RNA, small nuclear RNA, ribosomal RNA, transfer RNA, cryptic unstable transcripts. The RNA decay starts at the 3' end. The human exosome structure is globally the same except for the existence of three paralogs of Rrp44 called DIS3, DIS3L1 and DIS3L2. (*1,2*) (Table 1, Figure 1). Until 2012, only auto immune diseases were linked to the RNA exosome (*3*). Since, two human Mendelian diseases: syndromic diarrhea/ tricho-hepato-enteric syndrome (SD/THE) and Ponto-cerebellar hypoplasia type 1 (PCH1) were linked to the RNA exosome or its cofactor's defect. These diseases emphasize the importance of the RNA exosome in humans but their distinctive phenotypes with few

**Address correspondence to:*
Dr. Alexandre Fabre, Service de pédiatrie Multidisciplinaire, Hôpital des Enfants de la Timone, APHM, Marseille, France.
E-mail: Alexandre.fabre@ap-hm.fr

overlaps of clinical signs show that an alteration of the function of the RNA exosome can be quite segmentary, probably reflecting the various exosome substrates and functions.

2. Human disease related to the Ski complex

The Syndromic diarrhea/ Tricho hepato enteric (SD/THE) (OMIM : #222466, #614602): SD/THE has been linked to the alteration of *TTC37* and of *SKIV2L*, the orthologs of the yeast's Ski2 and Ski3, two components of the SKI complex (*4-6*). The SKI complex is an obligatory co-factor of the RNA exosome in the cytoplasm in yeast. To date, about 50 patients have been described (*7*). The main clinical features are an intractable diarrhea of infancy requiring parenteral nutrition, facial dysmorphism with a prominent forehead, a broad nose and hair abnormalities (wooly, easily removable), an intra uterine growth restriction, an immune deficiency (lack of immunoglobulin or

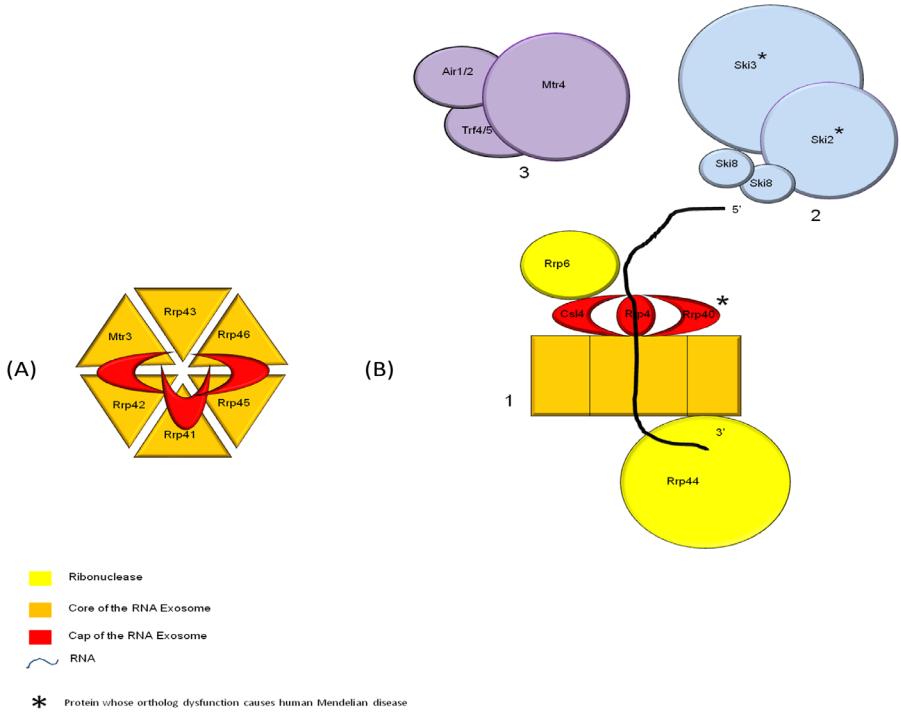

Figure 1. Model of yeast RNA exosome and cofactor. (A), Transversal view of RNA exosome. **(B)**, Front view of RNA exosome (**1**), SKI complex (**2**), and TRAMP complex (**3**).

Table 1. Proteins and genes of the RNA exosome and cofactor in human and yeast

Items	Yeast protein (*S. cerevisiae*)	Human protein	Human gene	Size of the protein (Human, AA)
Cap	Csl4/Ski4	Exosome complex component CSL4	*EXOSC1*	195
	Rrp4	Exosome complex component RRP4	*EXOSC2*	293
	Rrp40	Exosome complex component RRP40	*EXOSC3*	275
Core	Rrp41/Ski6	Exosome complex component RRP41	*EXOSC4*	245
	Rrp46	Exosome complex component RRP46	*EXOSC5*	235
	Mtr3	Exosome complex component MTR3	*EXOSC6*	272
	Rrp42	Exosome complex component RRP42	*EXOSC7*	291
	Rrp43	Exosome complex component RRP43	*EXOSC8*	276
	Rrp45	Exosome complex component RRP45	*EXOSC9*	456
	Rrp6	Exosome component 10	*EXOSC10*	885
	Rrp44/Dis3	Exosome complex exonuclease RRP44	*DIS3*	958
		DIS3-like exonuclease 1	*DIS3L1*	1054
Ski Complex	Ski2	Helicase SKI2W	*SKIV2L*	1246
	Ski3	Tetratricopeptide repeat protein 37	*TTC37*	1564
	Ski8	WD repeat-containing protein 61	*WDR61*	305
Tramp Complex*	Mtr4	Superkiller viralicidic activity 2-like 2	*SKIV2L2*	1042
	Air1	Zinc finger CCHC domain-containing protein 7	*ZCCHC7*	543
	Trf4	PAP-associated domain-containing protein 5	*PAPD5*	572

*The role of the TRAMP complex seems to differ between yeast and human.

absence of vaccine response). In half of the cases, liver abnormalities and skin abnormalities (mostly café au lait spots) are observed. Rarely, a congenital heart defect and platelet abnormalities are associated. The mortality is high with one third of the children deceased before 10 years. About half of the children can be weaned off parenteral nutrition and nearly all have a small size below the 3rd percentile (*8*). The phenotypes resulting from an alteration of either *SKIV2L* or *TTC37* cannot be differentiated, suggesting that it is the absence of the SKI complex which is responsible for the disease. As the SKI complex is linked to a degradation of mRNA in yeast it could be assumed that SD/THE is caused either by the presence of aberrant mRNA or the stabilization of normal transcripts which should be degraded. The clinical management is essentially supportive with nutritional support (mainly parenteral nutrition) and immunoglobulin supplementation.

3. Core RNA Exosome linked human disease

Pontocerebellar hypoplasia type 1 (PCH1) (OMIM #614676): PCH1 has been linked to mutation of *EXOSC3* encoding human RRP40, a protein of the cap of the RNA exosome (*9*). PCH is a group of inherited progressive neurodegenerative disorders with seven subtypes identified to date. All PCH associates atrophy of the pons and of the cerebellum and progressive microcephaly with developmental delay. PCH1 presents the distinctive feature of muscle atrophy secondary to spinal anterior horn cell loss which can lead to hypotonia and respiratory deficiency (*10*). Some patients develop growth retardation (*9*). *EXOSC3* mutations are found in 37% of PCH1 patients (*11*). Patients harbouring mutation in *EXOSC3* differ from other PCH1 patients by a longer lifespan, abnormal oculomotor function, and with time could appear a respiratory deficiency. Nearly 50 patients with a mutation in *EXOSC3* have been described since 2012 (*9,11-13*). Most of the patients present two missense mutations or, more rarely, a compound heterozygosity associating a missense and a null mutation. To date, no patient with homozygous null mutation has been reported. As some level of hypomorphy of the missense mutations exists, it seems that a total lack of RRP40 is not viable. There is some degree of genotype/phenotype correlation: indeed patients with homozygous missense mutation have a milder course, contrary to patients with null mutation and missense mutation. A clinical form with a milder severity has also been described: it associated spasticity, mild intellectual retardation and cerebellar atrophy, and is caused by compound missense mutations (*14*). The clinical management of the patient is symptomatic with nutritional support, and management of dyskinesia, dystonia and seizure (*10*).

4. Discussion

To date, two very different diseases have been related to a defect of the RNA exosome, one concerning the core exosome and the other, one of its co-factors. Except for the short stature found both in SD/THE and in PCH1, there are no shared clinical signs (Table 2). This could be related to an alteration of different types of RNA degradation. Indeed, the RNA exosome is implicated in different RNA degradation pathways: mRNA, Sn RNA, ribosomal RNA, transfer RNA, cryptic unstable transcripts. The ski complex is only necessary for the mRNA degradation (*15*) whereas RRP40 is part of the core of the RNA exosome and is involved in all the RNA exosome functions. Most of the genes causing the other subtypes of PCH are related to the Transfer RNA pathway (*10*). In PCH1, there are no cases of homozygous null mutation and, at least for one mutation, the RNA accumulation typical of exosome knock-out has not been observed (*9*), suggesting that some level of the exosome activity is conserved in PCH1 and that RRP40 is mainly necessary for the transfer RNA degradation, at least in humans. In 2012, the Perlman syndrome, an association of a congenital overgrowth syndrome with tumour susceptibility, was related to *DIS3L2* defect (*16*).

Table 2. Clinical sign Frequency of Syndromic diarrhea/Tricho-hepato-enteric syndrome and Pontocerebellar hypoplasia type 1with mutation in Exosc3

Items	Syndromic diarrhea/Tricho-hepato-enteric syndrome	Pontocerebellar hypoplasia type 1with mutation in *EXOSC3*
Nearly constant (> 90%)	• Intractable diarrhoea • Facial dysmorphism • Hair abnormality • IUGR • Imunodeficiency	• Important developmental delay • Muscle tone abnormal • Pons and/or cerebellar abnormalities
Frequent (50-90%)	• Skin abnormalities • Liver disease • Short stature despite adequate nutrition	• Progressive microcephaly • Respiratory insufficiency • Feeding difficulties
Inconstant (< 50%)	• Congenital cardiac defects • Platelet anomaly	• Epilepsy • Failure to thrive • Short stature • Abnormal oculomotor function

Dis3L2 is an exoribonuclease degrading cytoplasmic mRNA specially uridylated in an exosome-independent pathway (*17,18*). Thus these findings confirm the importance of a controlled homeostasis of RNA product and the potential consequences of imbalance.

In conclusion, to date, two very different human Mendelian diseases have been related to a dysfunction of the RNA exosome. They illustrate the versatility of the RNA exosome function and substrates. The question remains as to whether more diseases human Mendelian could be related to the dysfunction of the RNA exosome. Since non-functional exosome seems not to be viable, only moderate dysfunctions of the RNA exosome or cofactor alterations could be involved.

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Original Article

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Effects of targeted modulation of miR-762 on expression of the *IFITM5* gene in Saos-2 cells

Xinkai Mo[1,2], Yanqin Lu[1], Jinxiang Han[1,*]

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

Summary **Interferon-induced transmembrane protein 5 (IFITM5) is an osteoblast-specific membrane protein that plays an important role in the mineralization of the matrix in mature osteoblasts. However, understanding of the regulatory mechanism of IFITM5 expression is limited. Emerging evidence indicates that microRNAs (miRNAs) act as pivotal regulators in various biological processes including osteoblast proliferation and differentiation. This study aimed to investigate the impact of miRNAs on IFITM5 expression. Bioinformatic analyses predicted that miR-762 would be a potential regulator of *IFITM5*. A Dual-Luciferase Reporter Assay System indicated that miR-762 could bond with the 3'untranslated region (3'UTR) of *IFITM5 via* wild-type or mutant recombinant vectors and Western blotting verified that miR-762 negatively regulated IFITM5 expression. Collectively, these data indicate that miR-762 is a novel regulator of *IFITM5* and that it suppresses the expression of IFITM5 in Saos-2 cells.**

Keywords: *IFITM5* gene, miR-762, mineralization, target gene

1. Introduction

MicroRNAs (miRNAs) are an abundant class of endogenous, small (22-nucleotide), single strand, and noncoding RNA molecules that can bind to the 3'untranslated region (3'UTR) of target mRNA and regulate the stability and translation of mRNA, resulting in either inhibition of translation or degradation of the target mRNA (*1-3*). MiRNAs play essential roles in diverse biological processes, including cell proliferation, differentiation, apoptosis, and tumor oncogenesis (*4-6*). Recently, numerous studies have revealed that miRNAs play critical roles in osteoblast differentiation. For example, miR-34s reportedly inhibits osteoblast differentiation by targeting special AT-rich sequence-binding protein 2 (SATB2) (*7*). MiR-210 is upregulated during BMP4-induced osteoblastic differentiation of bone marrow stromal cells, promoting osteoblastic differentiation by downregulating activin A receptor type IB (Acvr1b) expression (*8*). MiR-141 and miR-200a are involved in preosteoblast differentiation through the translational repression of distal-less homeobox 5 (*Dlx5*), a bone-generating transcription factor expressed in preosteoblast differentiation (*9*).

Interferon-induced transmembrane protein 5 (IFITM5) is a member of the interferon-induced transmembrane (IFITM) protein family, of which there are at least four closely related members in humans (*IFITM1*, *-2*, *-3*, and *-10*) clustered on chromosome 11 (*10-12*). *IFITM5* encodes a 132-amino acid protein that has two transmembrane domains, such that it has extracellular N and C termini and an intracellular loop (*13*). IFITM5 has an aspartate-rich domain in the C-terminal region, that may be involved in calcium binding (*10*). Previous studies have confirmed the expression of IFITM5 in bone tissues in humans and that expression increases under culture conditions favoring osteogenic differentiation (*14-16*). Moreover, *IFITM5* is a positive modulator of mineralization according to overexpression and knockdown studies

**Address correspondence to:*
Dr. Jinxiang Han, Key Laboratory for Rare Disease Research of Shandong Province, Key Laboratory for Biotech Drugs of the Ministry of Health, Shandong Medical Biotechnological Center, Shandong Academy of Medical Sciences, Ji'nan, Shandong 250062, China.
E-mail: samshjx@sina.com

in cultured osteoblasts (*13*). Little is presently known about regulation of the *IFITM5* gene. The aim of the current study was to identify appropriate miRNAs and investigate their impact on IFITM5 expression. In human osteosarcoma Saos-2 cells, IFITM5 expression is closely correlated with differentiation and mineralization *in vitro* (*13*), so Saos-2 cells were chosen as a model of pro-mineralizing cells. Results revealed miR-762 can directly target the mineralization-related gene *IFITM5* and inhibit its expression, providing a theoretical basis for further study of the mechanisms by which miR-762 regulates the progress of bone mineralization.

2. Materials and Methods

2.1. *Cell culture and miRNA mimics transfection*

Human osteosarcoma Saos-2 cells, acquired from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), were cultured in McCoy's 5A (Gibco, Carlsbad, CA, USA) supplemented with 15% (v/v) fetal bovine serum (Gibco, Carlsbad, CA, USA). The cells were incubated at 37°C in a humid chamber containing 5% CO_2. MiRNA mimics and miRNA negative control (miR-NC) were purchased from a commercial manufacturer (GenePharma, Shanghai, China). Saos-2 cells were seeded in a 6-well plate (2×10^5/well) 24 h before transfection and cells were transfected with miRNA mimics (50 nM) or miR-NC (50 nM) using FuGENE® HD transfection reagent (Promega, Madison, WI, USA) in accordance with the manufacturer's protocol. The empty pmirGLO vector served as a blank control.

2.2. *Bioinformatic prediction*

MiRNAs can be predicted using a computational approach. First, the potential binding sites in the messenger RNA 3'UTR were identified according to specific base-pairing rules, and second, cross-species conservation requirements were implemented. A predictive search for miRNAs targeting *IFITM5* was performed using the programs TargetScan (*http://www.targetscan.org*), miRanda (*http://www.microrna.org*) and DIANA-microT (*http://diana.cslab.ece.ntua.gr*).

2.3. *Construction of plasmids and luciferase activity assay*

IFITM5 3'UTR including the predicted binding site of miR-762 was amplified by a reverse transcriptase-polymerase chain reaction (RT-PCR) (Table 1) and inserted into multiple cloning sites of the T-Vector pMD19 (pMD19-UTR) (Takara Bio, Otsu, Japan) using the *Sac*I and *Xba*I restriction sites. A site-directed gene mutagenesis kit (Takara Bio) was used to construct a mutant type of miR-762-binding site vector (pMD19-mUTR) with 4 base mutations within the seed region in accordance with the manufacturer's protocol. The pmirGLO dual-luciferase miRNA target expression vector (pmirGLO vector) containing both the firefly luciferase gene and Renilla luciferase gene was purchased from Promega (Madison, WI, USA). The particular restriction enzyme fragment of pMD19-UTR and pMD19-mUTR wsa inserted into 3'UTR down-stream of the firefly luciferase gene of the pmirGLO vector (pmirGLO-UTR and pmirGLO-mUTR). Both constructs were confirmed by restriction enzyme digestion and sequencing (Huada, Beijing, China). Saos-2 cells were seeded in a 96-well plate (2×10^4/well) 24 h before transfection. The cells were transfected with 50 nM of miRNA mimics or miR-NC and 1.0 μg/mL of reporter vectors using FuGENE® HD transfection reagent. Luciferase activity was measured 24 h after transfection using a Dual-Glo luciferase assay system (Promega), and firefly luciferase activity was

Table 1. Summary of sequences of primers used in this study

Usage	Gene	Direction	Sequence(5'→3')
Cloning[a]	*IFITM5*	Forward	CGAGCTCCAGGCTGGGTCCTGATCTGGGGC
		Reverse	GCTCTAGACTGGAACCAGGCACTTTTAAT
Mutagenesis	*IFITM5*	Forward	TGATCCTGGGGCCCTCCTAATCCAACATGGGCAC
		Reverse	GGATGGGGCAGGGATGGAGGCCCCACAGAAGGAG
qRT-PCR	*IFITM5*	Forward	TTGATCTGGTCGGTGTTCAG
		Reverse	GTCAGTCATAGTCCGCGTCA
	GAPDH	Forward	CACCATCTTCCAGGAGC
		Reverse	AGTGGACTCCACGACGTA
	miR-762	Forward	ATTATGGGGCTGGGGCCGGG
	U6 snRNA	Forward	CTCGCTTCGGCAGCACA
		Reverse	AACGCTTCACGAATTTGCGT

IFITM5: interferon-induced transmembrane protein 5; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR: quantitative RT-PCR; [a] *Sac*I and *Xba*I sequences were introduced into the cloned PCR product for subsequent subcloning.

normalized to Renilla luciferase activity. The empty pmirGLO vector served as a blank control, and values for cells with empty pmirGLO vector were set equally to 1 for each comparison. Experiments were performed in triplicate and repeated three times. Data are presented as means ± S.D.

2.4. *Total RNA extraction and real-time quantitative RT-PCR (qRT-PCR) analysis*

RNA was isolated using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol, and the yield and quality of RNA samples were determined with a NanoDrop 2000c spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Levels of expression were measured using qRT-PCR. For miRNA quantification, the first-strand miRNA-cDNA PCR template was generated from 1.0 μg of total RNA using a miRNA first-strand cDNA synthesis kit (Tiangen, Beijing, China) in accordance with the manufacturer's instructions. Using cDNAs as templates, qRT-PCR was performed with a LightCycler 480 II (Roche, Basel, Switzerland) and miRcute miRNA qPCR detection kit (Tiangen) in accordance with the manufacturer's instructions. Cycling conditions were 1 cycle of 94°C for 2 min and 40 cycles of 94°C for 20 s and 60°C for 34 s. For measurement of the *IFITM5* transcript from total RNA, total cDNA was synthesized using a reverse transcription kit (Takara Bio). QPCR was performed using SYBR Green II Master Mix (Roche) and the LightCycler 480 II. Amplification conditions were 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing 60°C for 20 s and extensionat 72°C for 30 s. All PCR assays were performed in triplicate. U6 *snRNA* and *GAPDH* were used as endogenous controls for miRNA and mRNA, respectively. The sequences of all primers are shown in Table 1. The $-\Delta\Delta Ct$ method was used to determine relative quantitation of miRNA and mRNA expression in Saos-2 cells, and the fold change was determined using the formula $2^{-\Delta\Delta Ct}$.

2.5. *Western blotting*

Eight days after transfection with miR-762 mimics or miR-NC, total protein extracts from the cells were homogenized in radio immunoprecipitation assay (RIPA) Lysis buffer (Beyotime, Shanghai, China) supplemented with phenylmethanesulfonyl fluoride (Beyotime, Shanghai, China) on ice for 60 min and then centrifuged at 14,000 g for 15 min at 4°C. The protein concentration was determined using the Bradford method (BioRad Laboratories, Carlsbad, CA, USA). Samples (20 μg) were suspended in Laemmli loading buffer and incubated at 100°C for 6 min. Proteins were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and was then transferred onto a polyvinylidene fluoride (PVDF) membrane and blocked by incubation with 5% low fat milk in TBST (10 mM Tris, 100 mM NaCl, and 0.05% Tween-20) for 1 h. The membranes were incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-human IFITM5 (1:1,000) from Sigma (St. Louis, MO, USA) and rabbit polyclonal anti-human GAPDH (1:2,000) from Proteintech Group, Inc. (Wuhan, Hubei, China). Unbound antibody was removed by washing with TBST buffer three times (10 min/wash). The membranes were then incubated with horseradish peroxide-conjugated secondary antibody for 1 h at room temperature, after which they were washed with TBST buffer three times (10 min/wash). The blots were developed with ECL reagent (Millipore Corporation, Billerica, MA, USA) in accordance with the manufacturer's instructions.

2.6. *Statistical analysis*

For quantitative data, results are expressed as the mean ± S.D. of *n* observations. Statistical significance between groups was determined using an unpaired Student's *t*-test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. *MiR-762 was predicted to bind to IFITM5 3'UTR*

To explore the regulatory mechanism of *IFITM5*, a regulator of osteoblast differentiation, bioinformatic analyses were performed using TargetScan, miRanda, and DIANA-microT to predict the putative miRNAs binding to *IFITM5* 3'UTR. Although each program predicted dozens of different miRNAs, the common miRNA they all predicted was miR-762. The programs all predicted that there would be one binding site in *IFITM5* 3'UTR. Further analysis revealed that 12 bases of the *IFITM5* 3'UTR gene matched the miR-762 seed sequence, in which 7 bases were highly conserved, suggesting that *IFITM5* may be a predicted target gene of miR-762 that is highly conserved (Figure 1A). A recent study found that miR-762 was involved in calcification of human vascular smooth muscle cells (VSMCs) calcification (*17*). Therefore, miR-762 was selected for further study.

3.2. *MiR-762 directly targeted IFITM5 3'UTR*

To confirm that *IFITM5* 3'UTR was a direct target of miR-762, a pmirGLO-UTR vector containing the miR-762 binding sites was constructed to perform a reporter assay, and pmirGLO-mUTR containing mutant binding sites was used as a control. Both constructs were confirmed by restriction enzyme digestion and sequencing (Figure 2). The luciferase activity of the reporter vectors was assayed 24 h after cotransfection

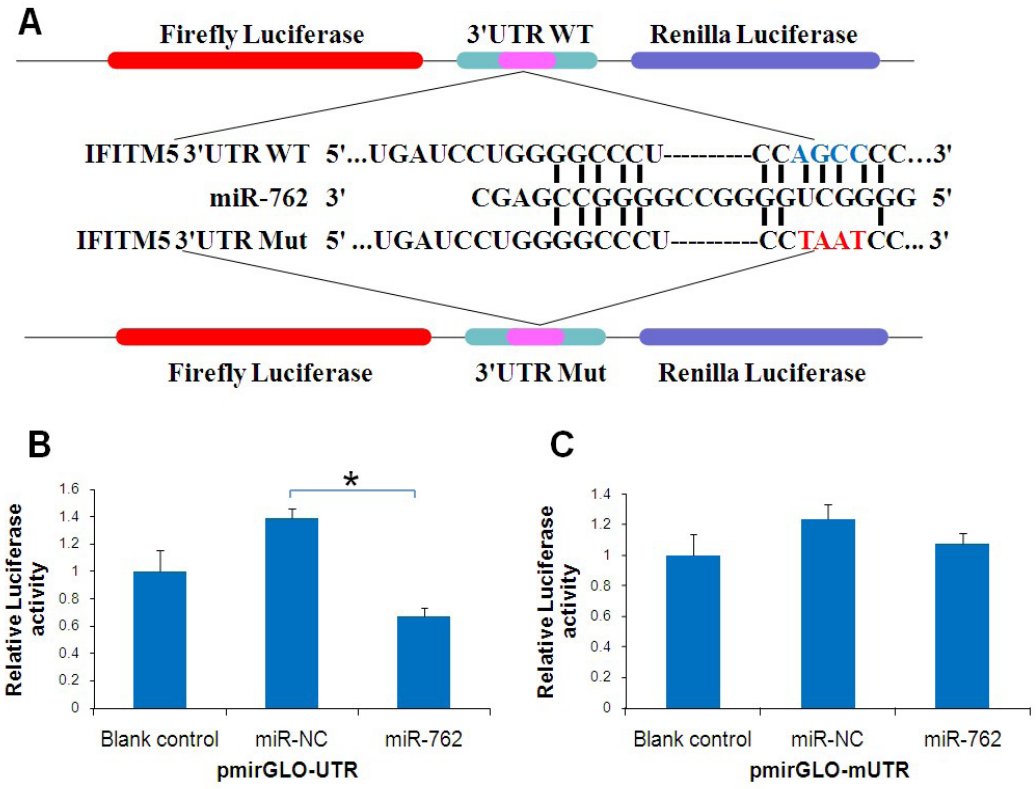

Figure 1. MiR-762 targets the 3'UTR of the *IFITM5* gene in Saos-2 cells. (A) Schematic of the *miR-762* putative target sites of *IFITM5* 3'UTR and alignment of *miR-762* with the seed sites of wild-type *IFITM5* 3'UTR and mutant *IFITM5* 3'UTR. The 4 mutated nucleotides are red, and the corresponding wild-type nucleotides are blue. **(B, C)** Luciferase assay. Saos-2 cells were transiently transfected with a pmirGLO, pmirGLO-UTR, or pmirGLO-mUTR vector, each with or without cotransfection with 50 nM of miR-762. After 24 h, reduced luciferase activity was observed after cotransfection of pmirGLO-UTR vector with miR-762 compared with those transfected with miR-NC, but not pmirGLO-mUTR. Data are presented as means ± S.D. from three independent experiments. * $p < 0.05$ *vs*. miR-NC.

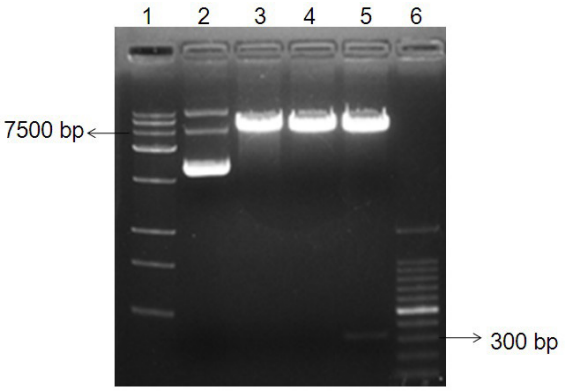

Figure 2. Restriction fragment analysis of the recombinant plasmid pmirGLO-UTR. The extracted recombinant plasmid was digested with two different restriction enzymes (*Sac*I and *Xba*I) and separated with a 0.8% agarose gel. The linear plasmid pmirGLO (at 7500 bp) and *IFITM5* 3'UTR (at 300 bp) are in lane 5. **Lane 1**: Trans15K DNA marker; **Lane 2**: undigested pmirGLO-UTR; **Lane 3**: *Sac*I digested pmirGLO-UTR; **Lane 4**: *Xba*I digested pmirGLO-UTR; **Lane 5**: *Sac*I and *Xba*I digested pmirGLO-UTR; **Lane 6**: 100 bp DNA ladder marker.

with miR-762 mimics or negative control in Saos-2 cells. When cotransfected with miR-762, the relative luciferase activity of pmirGLO-UTR was significantly suppressed by 52% (Figure 1B) in comparison to cotransfection with the negative control. In addition, the relative luciferase activity did not change when cotransfection was done with miR-762 and pmirGLO-mUTR containing a mutant binding site (Figure 1C), indicating that *IFITM5* 3'UTR is a direct target of miR-762.

3.3. *MiR-762 negatively regulated IFITM5 gene expression*

To further confirm that *IFITM5* is a target gene for miR-762, qRT-PCR and Western blotting analysis were used to detect the expression of IFITM5 regulated by miR-762 in Saos-2 cells. Levels of miR-762 and *IFITM5* mRNA in cells were determined using qRT-PCR. The expression of miR-762 was significantly up-regulated in comparison to miR-NC and the blank control in Saos-2 cells (Figure 3A). However, variations in the level of *IFITM5* mRNA were not noted ($p > 0.05$) (Figure 3B). Interestingly, the protein level of IFITM5 was suppressed by miR-762 mimics (Figures 3C and 3D) in comparison to miR-NC in Saos-2 cells. Taken together, these results suggest that *IFITM5* is a target gene of miR-762 and that IFITM5 is down-regulated by miR-762 only at the translational level in Saos-2 cells.

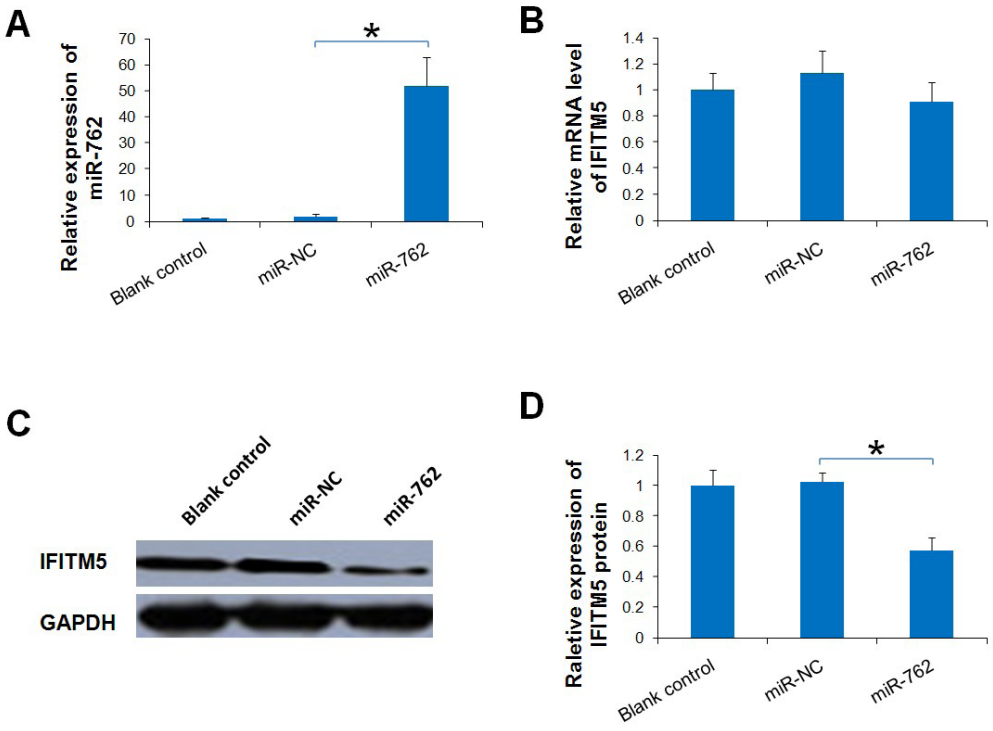

Figure 3. Forced expression of *miR-762* negatively regulated IFITM5 expression at the translational level. (A) Significantly upregulated expression of *miR-762* in Saos-2 cells transfected with 50 nM *miR-762* mimics was detected with qRT-PCR. *U6 snRNA* was the internal control gene. **(B)** *MiR-762* did not inhibit the expression of *IFITM5* at the mRNA level in Saos-2 cells. The mRNA expression of *IFITM5* and internal control *GAPDH* was detected with qRT-PCR. **(C**, **D)** Significantly downregulated expression of *IFITM5* at the translational level in Saos-2 cells was detected with Western blotting. The ratio of band intensity is relative to that of GAPDH. The band intensity was measured using ImageJ software. Data are presented as means ± S.D. from three independent experiments. * $p < 0.05$ *vs*. miR-NC.

4. Discussion

Bone homeostasis is balanced between bone formation and bone absorption by osteoblasts and osteoclasts, respectively (*18*). Therefore, study of osteoblast differentiation is essential to provide a better understanding of the skeletal disorders associated with bone mineralization, such as ankylosing spondylitis. MiRNAs display a novel mode of regulating gene expression and may regulate 30%-50% of human gene expression, though miRNAs account for only about 1% of all RNAs (*19*). Thus, important miRNAs should be identified as potential therapeutic targets for treatment of diseases related to bone mineralization.

Three different biological prediction programs (TargetScan, miRanda, and DIANA-microT) predicted that the 7 seed sequence of miR-762 combined with *IFITM5* 3'UTR at the same time. The 2-8 nucleotides of miRNA, known as the "seed region", are thought to be the most important for recognition (*20*). Comparison of the relative luciferase activity of the wild-type and mutant recombinant vectors to miR-NC revealed that miR-762 combines with *IFITM5* 3'UTR and then suppresses the expression of the target gene. There was a low level of miR-762 expression in Saos-2 cells and miR-762 was significantly up-regulated after miR-762 mimics were transfected. Furthermore, expression of IFITM5 was significantly down-regulated by over-expressed miR-762 at the translational level and not at the mRNA level. These findings suggest that miR-762 downregulated expression of IFITM5 *via* imperfect complementarity to *IFITM5* 3'UTR in Saos-2 cells.

MiR-762 is reportedly involved in tumorigenesis and in the development and progression of diseases such as diabetes and immune system diseases; miR-762 acts by targeting a number of important genes (*21-23*). Significance analysis of microarrays initially revealed that miR-762 is significantly upregulated in oral squamous cell carcinoma, suggesting that miR-762 plays a role in oral carcinogenesis (*21*). Recently, the neural precursor cell-enriched miR-762 was found to translationally downregulate adenosyl methionine decarboxylase 1 (Amd1), a key enzyme required for the synthesis of the polyamines spermine and spermidine, as it regulates both embryonic stem cell self-renewal and differentiation into a neural lineage (*24*). MiR-762 is also reported to be upregulated in human corneal epithelial cells in response to tear fluid and pseudomonas aeruginosa antigens and it negatively regulates the expression of innate host defense genes encoding RNase7 and ST2 (*23*). More importantly, Gui *et al*. (*17*) found that miR-762 disrupts calcium transport, thereby increasing the concentration of intracellular Ca^{2+} and thus resulting in Pi- and Ca-

induced human VSMC calcification both *in vivo* and *in vitro*. Interestingly, IFITM5 has an aspartate-rich domain in the C-terminal region that could be involved in calcium binding. IFITM5 overexpression in primary rat osteoblasts was found to result in a 60% increase in Ca^{2+} uptake (*13*). Hence, miR-762 is more likely to influence osteoblast mineralization through contact with *IFITM5*.

At present, two independent studies found that a mutation in the 5'UTR of the *IFITM5* gene is the cause of osteogenesis imperfecta (OI) type V (*14,25*). OI type V is characterized by an autosomal-dominant inheritance pattern, propensity to hyperplastic callus formation, and calcification of the fore-arm interosseous membrane (*26*). Research has fully demonstrated that *IFITM5* plays a major role in human skeletal physiology. Overexpression of IFITM5 in osteoblasts results in increased mineralization *in vitro* (*13*) and in the current study a low level of miR-762 in Saos-2 cells increased the expression of IFITM5, which may possibly contribute to hyperplastic callus formation. Furthermore, FK506-binding protein 11 (FKBP11) was identified as the only known binding partner of IFITM5 (*27*). Recent studies have revealed that the S-palmitoylation on IFITM5 promotes interaction with FKBP11 and cumulatively forms IFITM5-FKBP11-CD81, the prostaglandin F2 receptor negative regulator (FPRP) complex. Formation of this complex also leads to osteoblast-specific increased expression of 5 interferon-induced genes (*28-29*). Accordingly, speculation is that IFITM5 and FKBP11 might cooperatively regulate bone formation.

In conclusion, the current data indicate that miR-762 is a novel regulator of *IFITM5*. MiR-762 suppresses the expression of IFITM5 at the translational level in Saos-2 cells and probably contributes to the progression of bone mineralization. These findings shed new light on the roles of miRNAs in osteoblast differentiation.

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Case Report

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Auditory processing disorders associated with a case of Kartagner's syndrome

Jain Saransh*, Dwarkanath Mysore Vikas

JSS Institute of Speech and Hearing, Karnataka, India.

Summary **Kartagner's syndrome is a rare autosomal recessive disorder characterized by sinusitis, bronchiectasis and situs inversus. Otitis media is seen in 95% of the individuals with this syndrome due to recurrent respiratory infections and dysfunctional cilia in the middle ear. Earlier research reported the presence of structural and functional deficits in the auditory brainstem following long standing otitis media. However, no such findings have been reported in individuals with this syndrome. Thus, the present case report highlights the results of various audiological tests with special emphasis on investigating the auditory processing abilities in a known case of Kartagner's syndrome. In order to accomplish the aim, the audiological test battery was carried out on a 42 year old male patient diagnosed as having Kartagner's syndrome. The basic audiological tests, including immittance audiometry, pure tone audiometry, otoacoustic emission and auditory brainstem response (using click stimulus) results indicated the presence of mild to moderate mixed hearing loss in both ears. However, results of the auditory brainstem response (using speech stimulus) pointed toward abnormal speech processing skills. Thus, the behavioral test battery approach (including speech perception in noise test, gap detection test, temporal modulation transfer function test and duration pattern test) was followed and the findings suggested presence of auditory closure and temporal processing deficit. The outcome of the case study recommends that a complete test battery approach involving psychoacoustic tests should be used to assess such cases and auditory rehabilitation should be suggested accordingly.**

Keywords: Kartagner's syndrome, primary ciliary dyskinesia, auditory processing, psychoacoustics

1. Introduction

Kartagner's syndrome, also known as Primary Ciliary Dyskinesia (PCD) is a rare congenital disorder with an occurrence of approximately 1 in 30,000 live births (*1,2*) in the Western population. Although the true prevalence of the disease is unknown, the global prevalence data it is found to be 5 in 100,000 live births (*3*). The statistically extrapolated data from the global prevalence indicate that approximately 60,000 individuals in India are suffering from the disorder. The disorder was first described by Siewart (*4*), although Kartagner (*5*) was the first to describe the characteristics of the disease. The incidence of the disease was found to be more in males in comparison to females (*6*).

**Address correspondence to:*
Mr. Jain Saransh, JSS Institute of Speech and Hearing, Ooty Road, Mysore-25, Karnataka, India.
E-mail: saranshavi@gmail.com

Kartagner's syndrome is an autosomal recessive disorder (*7*) characterized by sinusitis, bronchiectasis and situs inversus (*8*). The main symptoms of the disease include chronic cough, chronic rhinitis, mucopurulant spertum, agenisis of the frontal sinus and other respiratory infections occurring during early childhood (*9*). Recurrent respiratory infections (*10*) and infertility (*11*) is commonly seen in adulthood. Otitis media is also commonly seen in this disorder due to recurrent respiratory infection and dysfunctional cilia in the middle ear (*12*).

Recurrent otitis media was reported in 95% of the individuals with PCD (*13*). Afzelius (*9*) also reported the presence of recurrent otitis media in these individuals. Long standing otitis media may result in structural and functional deficits in the auditory nervous system (*14*). However, no anatomical defects of the nervous system have been reported to be associated with Kartagner's syndrome. Yamashita *et al*. (*15*) reported the presence of amyotropic lateral sclerosis

in one of the patients suffering from Kartagner's syndrome. Olbrich *et al*. (*16*) also reported right-left asymmetry in such cases. In a review on the evaluation and management of PCD, Rossman and Newhouse (*17*) indicated that meningitis or brain abscess may occur as a consequence of sinusitis and bronchiectasis. The literature indicates that some form of anatomical and physiological deficits may be associated with Kartagner's syndrome, although, the investigation of auditory processing abilities has not been carried out in these individuals, as per the knowledge of the investigator. Thus, the present case report highlights the results of various audiological tests with special emphasis on investigating the auditory processing abilities in a known case of Kartagner's syndrome.

2. Case Report

A case aged 42 years/male reported to the department of audiology with a complaint of decreased hearing sensitivity in both ears. The case was referred from the department of otology and was diagnosed as having Kartagner's syndrome. Available reports of the oto-microscopic examination revealed subtotal tympanic membrane perforation and the presence of thick mucosa in the middle ear in both ears. Ear discharge was not present at the time of investigation. The case was diagnosed as having chronic suppurative otitis media with hereditary spherocytosis with Kartagner's syndrome. Available reports of Hematology showed the presence of spherocytes, polychromasia and anisocytosis in the semer. No other significant abnormality was noted and the features were consistent with hereditary spherocytosis. The x-ray investigation of the para nasal sinus revealed bilateral haziness of both maxillary sinuses, hypoplastic left frontal sinus and deviated nasal septum to the left side (Figure 1A). The ultrasonography test report for the abdomen revealed situs invertus and the chest x-ray revealed dextrocardia (Figure 1B). The report of the chest x-ray also showed abnormal lung thickening and thickened airway walls. The available result confirms the presence of Kartagner's triad (sinusitis, bronchiectasis and situs inversus) and hence the diagnosis.

A written consent was obtained from the patient in his native language (Kannada) prior to the evaluations. Detailed case history information obtained from the case reported the complaint of hearing loss in both the ears for the last 25 years. The case was an auto driver by profession and reported more difficulty in hearing while driving. The loss was slowly progressive in nature. The case history also pointed towards the complaint of ear discharge since childhood, which was mild, serous and intermittent in both ears. The frequent attacks of upper respiratory tract infection were also noted in the case history. There was no other significant history of ear pain, tinnitus or vertigo.

Acoustic Immitance measurements were carried out using an Interacoustics MT10 Immitance meter to investigate the presence of middle ear pathology and the results indicated a bilateral "B" type tympanogram, because of the subtotal perforation in both tympanic membranes. Distortion product and transient evoked oto-acoustic emission (Otoread, Interacoustics) were also absent (SNR < 6) within the frequency range of 700 Hz to 12 KHz. An audiometric measurement using a Maico MA53 dual channel diagnostic audiometer was carried out and the results indicated the presence of mild to moderate sloping mixed hearing loss (Table 1). Word recognition scores and speech identification

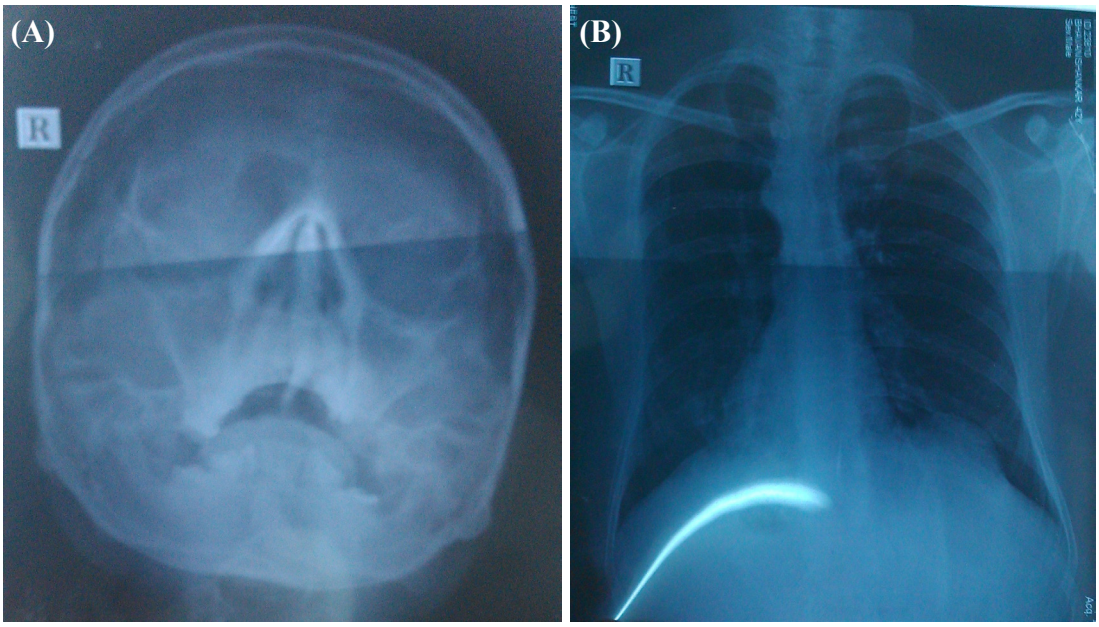

Figure 1. The X-Ray representation of (A) the para nasal sinuses; (B): the chest showing dextrocardia.

scores were consistent with the pure tone average.

The results were confirmed with the help of auditory brainstem responses (ABR). Click evoked ABR at the rate of 33.1/sec revealed presence of V peak at 55 dBnHL in right ear and 60d BnHL in left ear. Prolonged latencies of the III and V peak and delayed for I-III and I-V inter wave latency intervals were observed in both ears. The inter peak latency for the V peak was also found to be within normal range for different rate of stimulus presentation viz. 11.1/sec and 90.1/sec. The absolute latency and the inter wave latency difference of different ABR peaks at different rates of stimulus presentation is illustrated in Table 2.

The presence of ABR waveform indicated fair integrity of the auditory nerve and the lower auditory brainstem. In order to assess the integrity of the higher brainstem and the lower auditory cortex, middle latency response (MLR) and late latency responses (LLR) were measured using IHS Smart EP. The MLR was measured using click stimulus at the rate of 5.1/sec and the LLR was measured using click stimulus at the rate of 1.1/sec at a presentation level of 99 dBnHL. The results of MLR indicated a robust amplitude of the Na-Pa complex within normal absolute latency limits (Figure 2). The results of LLR also indicated the presence of the N1-P2 wave complex within normal absolute latency limits and good amplitude (Figure 3).

Vestibular evoked myogenic potentials (VEMP) were also administered using IHS Smart EP. Although VEMP is a neurophysiological technique to assess the functioning of the vestibular apparatus, the organs which are responsible for maintaining balance and equilibrium of the body in space, and the patient did not complain of any balance related problem, it is assessed because of the closed synchrony of the vestibular system with the hearing system. The results of the VEMP test indicated a prominent P13-N23 wave complex in both ears at an intensity of 107 dBnHL (Figure 4).

Speech evoked ABR using standardized "da" stimulus at an intensity of 95 dBnHL and a rate of 3.1/sec was also administered in the audiological test battery. The results of the speech evoked ABR were interesting with the presence of a V-A complex in

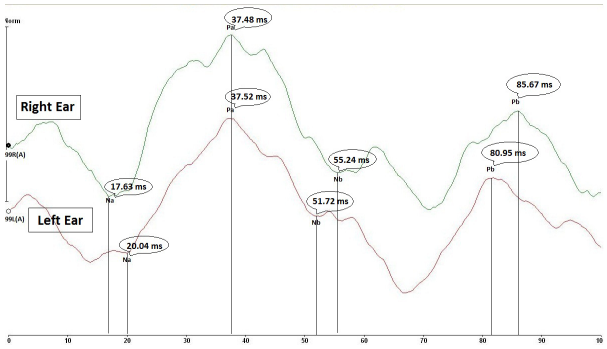

Figure 2. Middle Latency Responses. The waveform showing Na-Pa peak complex for the right and left ear.

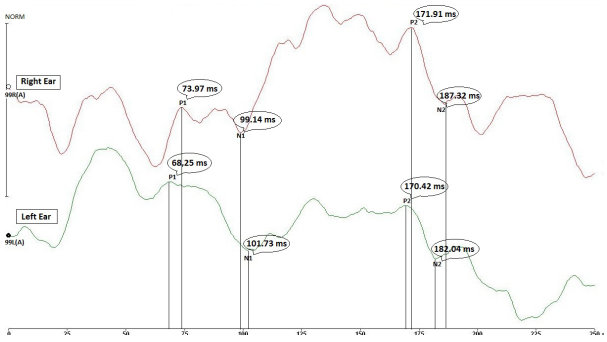

Figure 3. Late Latency Response. The waveform showing N1-P2 peak complex for the right and left ear.

Table 1. Audiometric findings for right and left ear for pure tone and speech

Procedure	Right Ear	Left Ear
PTA	33.3 dB	35 dB
SRT	35 dB	40 dB
SIS	90%	90%
MCL	85 dB	85 dB
UCL	>100 dB	>100 dB

Table 2. Latencies estimated from ABR test for both ears using various protocols

Parameters		Right Ear					Left Ear				
		Absolute Latency (in ms)			Inter-wave Latency Difference (in ms)		Absolute Latency (in ms)			Inter-wave Latency Difference (in ms)	
Intensity	Rate	I	III	V	III-V	I-V	I	III	V	III-V	I-V
90 dB	33.1/sec	1.37	3.75	5.89	2.14	4.52	1.44	3.78	6.09	2.31	4.65
80 dB	33.1/sec	1.56	3.94	6.52	2.58	4.96	1.69	3.86	6.38	2.52	4.69
70 dB	33.1/sec	NP	4.18	6.89	2.71	-	1.98	4.09	6.86	2.77	4.88
60 dB	33.1/sec	NP	NP	7.15	-	-	NP	NP	7.16	-	-
55 dB	33.1/sec	NP	NP	7.38	-	-	NP	NP	7.54	-	-
50 dB	33.1/sec	NP	NP	7.82	-	-	NP	NP	NP	-	-
45 dB	33.1/sec	NP	NP	NP	-	-	NP	NP	NP	-	-
90 dB	11.1/sec	1.29	3.62	5.77	2.15	4.48	1.39	3.56	5.98	2.42	4.59
90 dB	90.1/sec	NP	4.03	6.14	2.11	-	NP	NP	6.43	-	-

*NP: No identifiable peak observed.

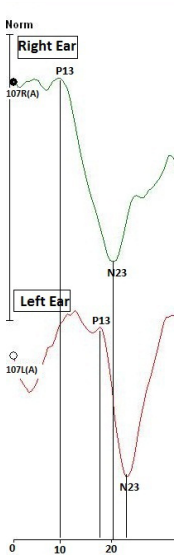

Figure 4. Vestibular Evoked Myogenic Potentials. The waveform is showing P13-N23 peak complex for the right and left ear.

both ears but absence of late peaks. These results were consistent across waveform replicability with the signal to noise ratio greater than '1' and the residual noise level less than 0.08 μV. These results were inconsistent with the other neurophysiological tests, however, but consistent with the patient's complaint of inability to understand speech clearly. Abnormal speech evoked ABR waveform suggests abnormal speech processing and the finding with a normal click evoked ABR is suggestive of possible auditory processing deficit (*18*). Thus, a detailed investigation of processing abilities was warranted to confirm the findings.

In order to investigate the presence of auditory processing abilities, a test battery approach was followed and tests like speech perception in noise (SPIN), gap detection thresholds (GDT), temporal modulation transfer function (TMTF) using 8 Hz modulation, 60 Hz modulation and 200 Hz modulation rates, and a duration pattern test (DPT) was administered.

SPIN was measured using standardized Kannada paired words (*19*) and monosyllables using a Maico MA53 dual channel diagnostic audiometer. The stimulus was presented using monitored live voice and the intensity level was kept constant at 10 dB above the most comfortable level in both the ears and the intensity of the speech noise was varied in such a way that the SNR was +10 dB, +5 dB, 0 dB and -5 dB. The results indicated more than 50% scores for both paired words and monosyllables at the SNR level of +10 dB and +5 dB, however, less than 50% scores were obtained for the SNR of 0 dB and -5 dB. The results for the paired words were consistently better than the monosyllables. The poor scores on the SPIN are indicative of abnormal auditory closure ability.

GDT was measured using a maximum likelihood procedure (MLP, a MATLAB based toolbox for psychoacoustic experiments) (*20*). The stimulus was presented using the personal computer routed *via* a Maico MA53 audiometer equipped with TDH 39 headphones. The intensity was monitored at the most comfortable level in both ears. The results indicated poor gap detection scores (9.13 ms) in comparison to normal (5 ms) (*21*) individuals. The poor scores on the GDT indicated abnormal temporal resolution abilities. The abnormal performance on the temporal resolution skills were also indicated by poor TMTF scores for 8 Hz, 60 Hz and 200 Hz. TMTF was also measured using MLP routed *via* audiometer with the stimulus presented at the most comfortable level in both ears. Results indicated -8 dB modulation detection thresholds at 8 Hz, -6 dB thresholds at 60 Hz and -2 dB thresholds at 200 Hz. These results were far poorer in comparison to normal individuals (-13 dB for 8 Hz, -9 dB for 60 Hz, and -4 Hz for 200 Hz) (*21*).

Finally, the DPT was also administered using MLP. A total of 30 stimuli using long and short stimulus in three tone patterns was presented. The patient's task was to indicate the correct sequence of stimulus presentation. Results of the test indicated very poor scores (16 correct pattern identification) in comparison to age matched normal individuals (28 correct pattern identification) (*21*). Poor scores on this test indicate abnormal temporal sequencing abilities.

Results of various auditory processing tests confirm the presence of abnormal auditory processing ability in the individual with Kartagner's syndrome.

3. Discussion

Kartagner's syndrome is a rare genetic disorder due to deficits in ciliary motion (*15*). Immotile ciliary action in Kartagner's syndrome is mainly manifested in the respiratory tract and the fallopian tube (*22*). As a result sinusitis and bronchiectasis are associated with the disorder. Defective ciliary rotation in these cases results in situs inversus (*23,24*). Recurrent otitis media are commonly seen in such individuals (*25*).

As Webster *et al*. (*14*) reported that long standing otitis media may result in structural and functional deficits in the brainstem and higher cortical structures, there is a need to investigate the presence of auditory processing disorders in individuals with Kartagner's syndrome. Moreover, significant hearing loss is also associated with recurrent otitis media (*26*). Thus, the present study aimed at investigating the audiological characteristics of a known case of Kartagner's syndrome with special emphasis on auditory processing disorders.

A test battery approach was used to assess the auditory functioning in the client. Acoustic Immitance measurement revealed a flat tympanogram with normal ear canal volume. Such findings are suggestive of a perforated tympanic membrane with active middle ear pathology (*27*). Acoustic reflex and otoacoustic

emissions were not obtained probably because of the perforated tympanic membrane (*28,29*). Audiometry reports indicated bilateral mild to moderate sloping mixed hearing loss with correlating speech scores. These findings were consistent with long standing otitis media (*30*).

ABR to click stimuli also indicated the presence of middle ear pathology in terms of prolonged absolute latencies and delayed inter wave intervals. Prolonged latencies of wave III and wave V was also reported by Folsom, Weber and Thompson (*31*) in individuals with recurrent otitis media. Gunnarson and Finitzo (*32*) and Hall and Grose (*33*) reported delayed interwave intervals for I-V and III-V wave associated with persistent recurrent otitis media. Thus, the findings for click evoked ABR was consistent with the literature findings. MLR and LLR were also administered and the findings pointed towards normal integrity of the auditory brainstem and lower auditory cortex. The presence of MLR and LLR peaks within normal absolute latency range in both ears (without delay caused due to the presence of middle ear pathology) may be due to the high level of stimulus presentation. Both MLR and LLR were administered at 99 dBnHL, which is approximately 60 dB above the pure tone average. With such a high intensity of stimulus presentation, attenuation caused due to the conductive component may be compensated.

The basic audiological findings suggested the presence of mixed hearing loss in the present individual. However, the results of speech evoked ABR were not consistent with the non speech findings. The results signify the presence of an auditory processing disorder and a detailed investigation was carried out using a set of psychoacoustic tests. The findings on the SPIN indicated the presence of auditory closure deficits, on GDT and TMTF pointed towards the presence of temporal resolution deficits and poor scores on the DPT showed existence of temporal sequencing deficits. These results confirmed the presence of an auditory processing disorder in the individual with Kartagner's syndrome, and thus, the detailed investigation should be carried out with each such individual and management considering the processing deficit should be provided.

4. Conclusion

Kartagner's syndrome is a congenital disorder characterized by the presence of sinusitis, bronchiectasis and situs invertus. Recurrent otitis media is common in these individuals and hence, it is likely that auditory processing disorders may be present in such individuals. The present study aimed at investigating audiological characteristics with special emphasis on auditory processing disorders in a known case of Kartagner's syndrome. A test battery approach was applied and results of basic audiological tests pointed towards the presence of bilateral mild to moderate mixed hearing loss. In order to assess auditory processing ability, a set of phychoacoustic tests were administered and the results showed the presence of auditory closure and temporal processing deficits.

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Case Report

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Pulmonary nocardiosis in patients with connective tissue disease: A report of two cases

Shinya Hagiwara[1], Hiroto Tsuboi[1], Chihiro Hagiya[1], Masahiro Yokosawa[1], Tomoya Hirota[1], Hiroshi Ebe[1], Hiroyuki Takahashi[1], Hiroshi Ogishima[1], Hiromitsu Asashima[1], Yuya Kondo[1], Naoto Umeda[1], Takeshi Suzuki[1], Shigemi Hitomi[2], Isao Matsumoto[1], Takayuki Sumida[1,*]

[1] *Department of Internal Medicine, Faculty of Medicine, University of Tsukuba, Tsukuba, Japan;*
[2] *Department of Infectious Diseases, Faculty of Medicine, University of Tsukuba, Tsukuba, Japan.*

Summary **Reported here are 2 patients with connective tissue disease who developed pulmonary nocardiosis. Case 1 involved a 73-year-old man with malignant rheumatoid arthritis treated with prednisolone 25 mg/day. Chest X-rays revealed a pulmonary cavity and bronchoscopy detected *Nocardia* species. The patient was successfully treated with trimethoprim/sulfamethoxazole. Case 2 involved a 41-year-old woman with systemic lupus erythematosus. The patient received remission induction therapy with 50 mg/day of prednisolone and tacrolimus. Six weeks later, a chest CT scan revealed a pulmonary cavity; bronchoscopy resulted in a diagnosis of pulmonary nocardiosis. The patient had difficulty tolerating trimethoprim/sulfamethoxazole, so she was switched to and successfully treated with imipenem/cilastatin and amikacin.**

Keywords: Connective tissue disease, immunosuppressive therapy, nocardia, pulmonary nocardiosis

1. Introduction

Nocardia is an aerobic gram-positive bacillus belonging to the family Nocardiaceae within the order Actinomycetales. It is weakly acid-fast and is widely distributed in soil. *Nocardia* species are usually considered to be opportunistic pathogens. A previous report showed that more than half of 1,000 patients with nocardiosis were immunosuppressed (*1*). Risk factors for nocardiosis include corticosteroid treatment, lymphoma, solid tumors, stem-cell or organ transplantation, human immunodeficiency virus (HIV) infection, and diabetes (*2*).

Connective tissue disease (CTD) is typically treated with corticosteroids and immunosuppressants and biologics. Nocardiosis is an occasional, but potentially serious, infection for immunosuppressed patients. Reported here are cases of 2 patients with CTD who developed pulmonary nocardiosis during immunosuppressive therapy.

**Address correspondence to:*
Dr. Takayuki Sumida, Department of Internal Medicine, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan.
E-mail: tsumida@md.tsukuba.ac.jp

2. Case reports

2.1. *Case 1*

A 73-year-old Japanese man was admitted to this hospital in May 2012 for polyarthritis. He tested positive for anti-citrullinated peptide antibody (anti-CCP antibody) and rheumatoid factor (RF) and he met the 2010 rheumatoid arthritis (RA) classification criteria (American College of Rheumatology/European League Against Rheumatism) (*3*). The patient was diagnosed with rheumatoid arthritis, and this condition was highly active. The patient had difficulty tolerating methotrexate (MTX) and corticosteroids presumably due to renal dysfunction and diabetes. Therefore, he was treated with adalimumab (ADA), which was subsequently switched to etanercept (ETN) because of primary failure (Figure 1A).

In September 2012, the patient was again admitted to this hospital because his arthritis had worsened. Blood examinations at admission showed decreased

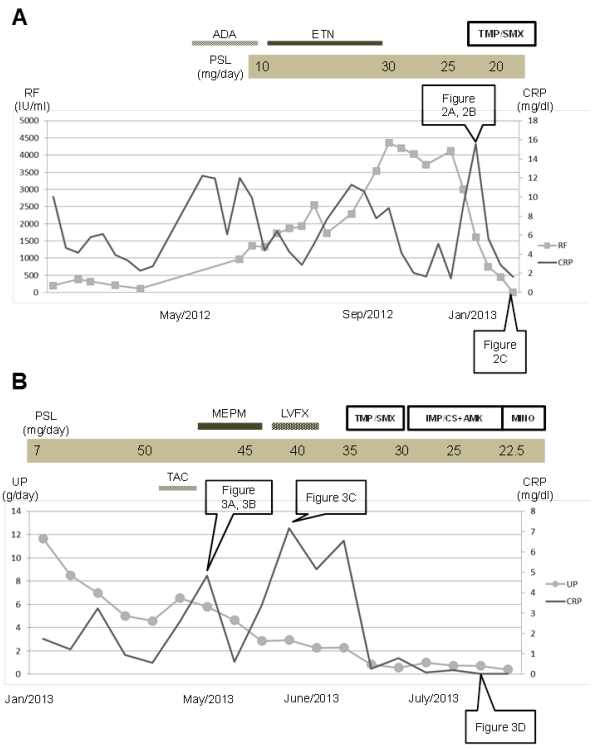

Figure 1. Clinical courses. (A) Case 1: Treatments and changes in levels of serum C-reactive protein (CRP) and rheumatoid factor (RF) are shown along with the timing of chest X-rays and computed tomography (CT) scans (*Abbreviations*: ADA, adalimumab; ETN, etanercept; PSL, prednisolone; TMP/SMX, trimethoprim/sulfamethoxazole; CRP, C-reactive protein; RF, rheumatoid factor; Sep, September; Jan, January); **(B)** Case 2: Treatments and changes in levels of serum C-reactive protein (CRP) and proteinuria are shown along with the timing of chest X-rays and computed tomography (CT) scans (*Abbreviations*: PSL, prednisolone; TAC, tacrolimus; MEPM, meropenem; LVFX, levofloxacin; TMP/SMX, trimethoprim/sulfamethoxazole; IPM/CS, imipenem/cilastatin; AMK, amikacin; CRP, C-reactive protein; UP, urine protein; Jan, January).

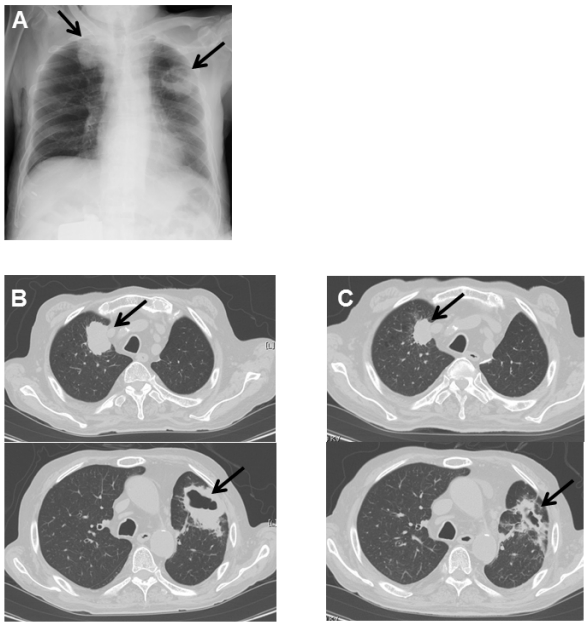

Figure 2. Chest X-ray and computed tomography findings in case 1. On admission, chest X-rays **(A)** and computed tomography (CT) **(B)** revealed a cavity in the upper left lung lobe and a mass lesion in the upper right lung lobe (indicated by arrows). Two weeks after initiation of trimethoprim/sulfamethoxazole (TMP/SMX), a chest CT **(C)** scan revealed marked reduction in the size of both the cavity and the mass lesion (indicated by arrows). **(A)** Chest X-ray on admission (January 2013). **(B)** Chest CT scan on admission (January 2013). **(C)** Chest CT scan 2 weeks after initiation of trimethoprim/sulfamethoxazole (TMP/SMX).

complement levels (CH50: < 10 U/mL; C3 75: mg/dL; and C4 7: mg/dL) as well as marked elevation of RF (4,356 IU/ml). Moreover, chest X-rays revealed bilateral pleural effusion, and thoracentesis confirmed exudative pleurisy without infection. Consequently, the presence of definite RA, low complement levels, and high titers of RF led to a diagnosis of malignant rheumatoid arthritis (MRA). The patient was treated with 30 mg/day (0.6 mg/kg/day) of oral prednisolone (PSL), and ETN was discontinued. His arthritis improved markedly, as did his pleurisy (Figure 1A).

In January 2013, he complained of general fatigue and fever, and he was admitted to this hospital, where he was treated with 25 mg/day of oral PSL. Chest X-rays and computed tomography (CT) revealed a cavity in the upper left lung lobe as well as a mass lesion in the upper right lung lobe (Figure 2A, 2B). Because pulmonary tuberculosis and mycosis were suspected, sputum and gastric fluid were cultured for *Mycobacterium* species and levels of beta-D-glucan in serum were determined. The results of these examinations were all negative. Therefore, bronchoscopy was performed to diagnose these lung lesions. *Nocardia* species were found in cultured bronchoalveolar lavage fluid (BALF) but not malignant cells. A systemic examination revealed no involvement of the brain or skin. Ultimately, lung nocardiosis was diagnosed. Moreover, the 16S ribosomal RNA genes of the recovered organisms were amplified using a polymerase chain reaction (PCR) as described previously (*4*), and the 1,367-nucleotide product was sequenced with an ABI PRISM 3130 genetic analyzer (Applied Biosystems Japan, Tokyo, Japan). Analyses with the Basic Local Alignment Search Tool showed that the sequence had 99.9% similarity to that of *Nocardia farcinica* ATCC 3318^T (GenBank accession number: Z36936).

Oral administration of trimethoprim/sulfamethoxazole (TMP/SMX) (960-4,800 mg/day) was started for lung nocardiosis, and symptoms such as fatigue and fever improved. Two weeks after the initiation of TMP/SMX, a marked reduction in the cavity and in the mass lesion in the lungs was noted on a chest CT scan (Figure 2C). TMP/SMX administration was continued for 6 months (Figure 1A).

2.2. *Case 2*

The patient in the second case was a 41-year-old woman who had been diagnosed with systemic

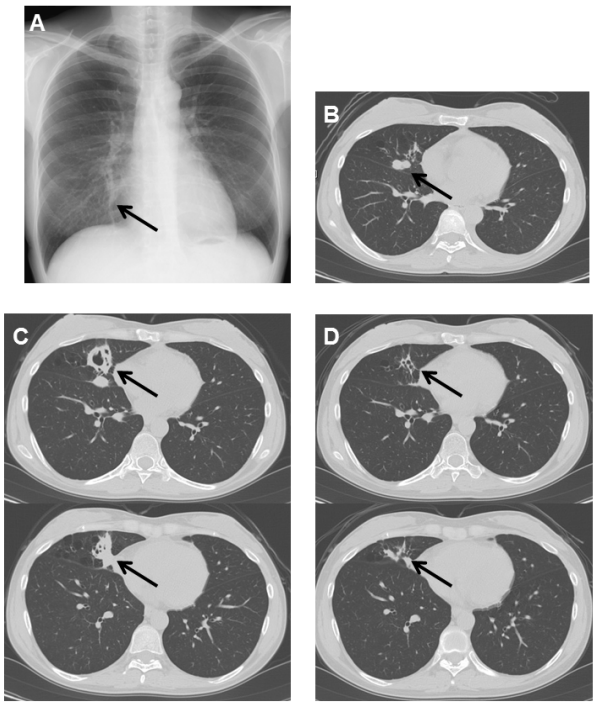

Figure 3. Chest X-ray and computed tomography findings in case 2. Two weeks after the initiation of remission induction therapy for systemic lupus erythematosus (SLE), chest X-rays **(A)** and computed tomography (CT) **(B)** revealed nodules in the middle right lung lobe (indicated by arrows). One month after the first chest CT scan, a second chest CT scan showed that the nodules had enlarged and formed a cavity **(C)** (indicated by arrows). After 6 weeks of treatment for nocardiosis, a chest CT scan **(D)** revealed that the cavity (indicated by arrows) was less opaque. **(A)** A chest X-ray 2 weeks after initiation of remission induction therapy. **(B)** Chest CT scan 2 weeks after initiation of remission induction therapy. **(C)** Chest CT scan 1 month after the first chest CT scan. **(D)** Chest CT scan after 6 weeks of treatment for nocardiosis.

lupus erythematosus (SLE) along with nephritis and serositis. The onset of SLE occurred at the age of 19, and the patient had received continued maintenance therapy with 7 mg/day of oral PSL for several years (Figure 1B).

In June 2012, proteinuria appeared, and edema in both legs gradually worsened. As a result, the patient was admitted to this hospital in January 2013. Proteinuria of 6 - 12 g/day, pericardial effusion, a high anti-DNA antibody titer according to radioimmunoassay (90 IU/mL), and a low white blood cell (WBC) count ($3,100/mm^3$) were observed. On the basis of these findings, a flare-up of SLE was diagnosed. She was again given remission induction therapy with 50 mg/day (1 mg/kg/day) of oral PSL and tacrolimus (TAC). Two months later, the proteinuria had decreased to 1 - 2 g/day (Figure 1B).

After TAC was started, a blood examination showed increased levels of C-reactive protein (CRP). Moreover, chest X-rays and CT scans showed nodules in the middle right lung lobe (Figure 3A, 3B). Her respiratory symptoms were very mild, and the sputum culture results were negative. Although antibiotics (meropenem and levofloxacin) were administered and TAC was discontinued, the CRP increased again after occasionally decreasing. One month after the first chest CT scan, a second chest CT scan was performed and showed that the nodules had enlarged and formed a cavity (Figure 3C). Bronchoscopy was performed, and *Nocardia* species were identified in the BALF culture. Thus, pulmonary nocardiosis was diagnosed (Figure 1B). Furthermore *Nocardia* species were identified as *Nocardia farcinica* by sequencing analysis of the 16S ribosomal RNA genes as was done in case 1.

Oral administration of TMP/SMX (1,440-7,200 mg/day) was started for pulmonary nocardiosis. The treatment with TMP/SMX was effective, and the serum CRP decreased dramatically after initiation of TMP/SMX. However, 2 weeks later, the TMP/SMX was switched to imipenem/cilastatin (IPM/CS) and amikacin (AMK) because the patient had hyponatremia. The treatment with IPM/CS and AMK was continued for 4 weeks, following oral administration of minocycline (MINO). Serum CRP remained low during these treatments (Figure 1B). After 6 weeks of the treatment (2 weeks with TMP/SMX and 4 weeks with IPM/CS and AMK), a chest CT scan showed a reduction in the pulmonary cavity (Figure 3D).

3. Discussion

Patients with CTD undergoing immunosuppressive therapy are at risk of acquiring various opportunistic infections, such as mycosis, *Pneumocystis jiroveci* pneumonia, and mycobacteriosis. Although nocardiosis is infrequent, some reports have noted nocardiosis in patients with CTD (*5,6*).

Pulmonary nocardiosis is the most common clinical presentation of nocardiosis. The onset of symptoms includes a productive or nonproductive cough, shortness of breath, chest pain, hemoptysis, fever, night sweats, weight loss, and progressive fatigue (*7*). Because nocardiosis does not have any specific symptoms, whether nocardia is present must be determined, such as by a culture test, in high-risk patients. Indeed, Martínez Tomás and colleagues showed that the mean time to diagnosis for nocardiosis was 42 days (*8*). Although a chest CT scan is useful at diagnosing pulmonary nocardiosis, a cavity opacity may not necessarily be observed. As potential findings include pleural effusion, multiple nodules, and chest wall extension (*9*).

Nocardia species must be identified for a definitive diagnosis of nocardiosis. Generally, a sputum culture or bronchoscopy (obtaining BALF) is performed to diagnose pulmonary nocardiosis. In the 2 current cases, *Nocardia* species were not detected in sputum cultures but were detected in BALF cultures. Importantly, a previous report showed that specimens obtained by invasive methods were required for definitive diagnosis in 47% of patients

with pulmonary nocardiosis (*8*). Moreover, Scott and colleagues reported that percutaneous transthoracic needle biopsy (PTNB) was useful at diagnosing patients with acquired immunodeficiency syndrome (AIDS) (*10*).

General treatment recommendations for nocardiosis are hindered by the lack of prospective controlled trials. Optimal antimicrobial treatment regimens have not been firmly established (*7*). TMP/SMX is most commonly used to treat nocardiosis (*7*,*11*). Alternative antimicrobial agents with activity against *Nocardia* species include AMK, IPM, meropenem, ceftriaxone, cefotaxime, MINO, moxifloxacin, levofloxacin, linezolid, tigecycline, and amoxicillin-clavulanic acid (*7*). Combination therapy with IPM and cefotaxime, AMK and TMP/SMX, IPM and TMP/SMX, AMK and cefotaxime, or AMK and IPM may provide increased effectiveness (*7*). Both of the current patients responded well to TMP/SMX monotherapy, but the patient in case 2 had to be switched from TMP/SMX to IPM/CS and AMK because of an adverse reaction following oral administration of MINO. Interestingly, that patient did not respond sufficiently to meropenem or levofloxacin.

When treating nocardiosis, drug resistance and the duration of the treatment are also important points to consider. Recent reports showed that 2% to 43% of *Nocardia* species were resistant to TMP/SMX (*11*). The duration of treatment is generally prolonged to minimize the risk of disease relapse. Immunocompetent patients with pulmonary or multifocal (non-central nervous system, CNS) nocardiosis may be successfully treated with 6 to 12 months of antimicrobial therapy. Immunosuppressed patients and those with CNS disease should receive at least 12 months of antimicrobial therapy with appropriate clinical monitoring (*7*).

The findings reported here indicate that if patients with CTD who are receiving immunosuppressive therapy have abnormal findings in the lungs (including pulmonary cavities), then the possibility of nocardiosis should be considered in addition to mycosis and mycobacteriosis. If patients have negative sputum cultures, bronchoscopy may need to be performed to obtain BALF in order to detect *Nocardia* species.

In conclusion, pulmonary nocardiosis is one of the important differential diagnoses for pulmonary lesions (including pulmonary cavities) as well as mycobacteriosis and mycosis in patients with CTD undergoing immunosuppressive therapy.

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Commentary

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Anti-cytokine treatment for Takayasu arteritis: State of the art

Enrico Tombetti[1,2,*], Maria Chiara Di Chio[1,2], Silvia Sartorelli[1,2], Enrica Bozzolo[1], Maria Grazia Sabbadini[1,2], Angelo A. Manfredi[1,2], Elena Baldissera[1]

[1] *Unit of Internal Medicine and Clinical Immunology, San Raffaele Scientific Institute, Milan, Italy;*
[2] *Università Vita-Salute San Raffaele, Milan, Italy.*

Summary **Takayasu arteritis (TA) is a rare and idiopathic large-vessel arteritis typically affecting young women which has important morbidity and mortality. There are no animal models of TA and pathogenesis is still mysterious. Clinical assessment lacks accurate activity indexes and is based on the integration of clinical, laboratory and radiological data. TA rarity has hampered randomized clinical trials and the achievement of high-quality evidence to guide clinical activity. Prevention of vascular progression, with progressive vessel wall remodelling and hyperplasia, is the main therapeutic goal. Medical therapy remains the mainstay of management and comprises traditional immunosuppressive agents and anti-inflammatory drugs, such as steroids and blockers of pivotal cytokines, TNF-α and IL-6. These strategies however only partially limit vascular progression, indicating that local molecular events are involved. Here we discuss recent data suggesting that selected cellular components of TA lesions should be evaluated as novel therapeutic targets.**

Keywords: Takayasu arteritis, tumor necrosis factor (TNF), tocilizumab, angioplasty, surgery

Takayasu arteritis (TA) is an idiopathic inflammatory disease, typically affecting young women which has important morbidity and mortality (*1-8*). Although TA is a systemic disease, inflammation primarily localizes in the large arteries, such as the aorta, the pulmonary artery and their major branches (*7,9*). Traditionally, the TA course is subdivided into the early, active phase and the late, chronic phase (*10*). Although the active phase can be mono- or oligo-phasic, a chronic-relapsing course is more common, needing long-term treatment (*1,3,7*).

The outcome of the deregulated inflammatory process can be an irreversible fibrosis of the vessel wall. On occasion, vasculitis can result in aneurism formation secondary to the action of various enzymes involved in extracellular matrix remodelling, such as metalloproteinases. Although aneurysms are reported in about 10-25% of patients, stenotic or occlusive lesions occur in more than 90% (*1,5,7,11-14*). In addition to *sequelae* associated with cerebral, organ and limb ischemia and to expanding aneurysms, patients develop arterial hypertension, accelerated atherosclerosis and heart failure.

TA is a rare and truly orphan disease: ample areas of TA pathogenesis and disease activity assessment are still poorly known. There are no animal models of the disease. Studying TA is further complicated by difficulties in obtaining tissues from living patients. Histology reveals focal panarteritis with macrophages, lymphocytes and dendritic cells, frequently in a granulomatous organization (*15*). The local production of growth factors, including platelet-derived growth-factor (PDGF) and vascular endothelial growth factor (VEGF), drives local vessel wall changes responsible for arterial thickening and progressive lumen narrowing (*16*).

In the absence of accurate markers, activity definition is based on the integration of clinical, laboratory and radiological data and it is frequently established a *posteriori* on an already-occurring progression of vascular involvement as assessed by radiologic or clinical evaluation. To date, our capacity to identify the processes undelaying vascular progression is very poor ((*7*) and unpublished data).

TA rarity has prevented controlled comparative clinical trials. Accordingly, the evidence to guide clinical management is poor (*17*) and no definitive

**Address correspondence to:*
Dr. Enrico Tombetti, San Raffaele University Hospital, Unit of Internal Medicine and Clinical Immunology, Via Olgettina 60, Milano 20132, Italy.
E-mail: tombetti.enrico@hsr.it

treatment advice can be given as to dosing, duration and choice of therapeutic agents. Clinical or laboratory systemic inflammatory response is variably associated with disease activity and thus it cannot be used as a reliable therapeutic target. Vascular complications are the events that most clearly impinge on the clinical outcome (*5*). Therefore we believe that the main therapeutic goal in TA is prevention of vascular progression through control of vascular inflammation and remodelling. Accordingly, vascular imaging is crucial for monitoring TA course. There is no single imaging modality that can provide all the information required and each method has distinct and complementary roles in monitoring disease activity (*18*).

TA treatment is based on medical and operative (endovascular or surgical) therapies (*19*). Medical therapy is the cornerstone of early, active TA and it is primarily based on high-dose corticosteroids (*19,20*). Unfortunately, 55-90% of patients relapse when corticosteroids are tapered and need to enhance steroid and immunosuppressive treatment (*1,4,7,21,22*).

The addition of immunosuppressive agents (methotrexate, azathioprine, mycophenolate mofetil and leflunomide) often allows better disease control and further steroid reduction in steroid-resistant or -dependent patients. Nonetheless, clinical relapses and progression of vascular involvement remain frequent (*19*). Patients with uncontrolled disease with immunosuppressive agents can benefit from biologic therapy. Tumor Necrosis Factor-α (TNF)-inhibitors are the most widely studied biologic agents in TA. TNF is involved in granuloma formation and blood levels of TNF are increased in TA patients (*23*). In five major observational uncontrolled cohorts, TNF-inhibitors were studied in overall 90 refractory TA patients (*24-28*). Complete or partial response was observed in 85-90% of patients, with a relapse rate of 33-60%. Four studies documented radiological follow-up, although with different modalities, and new lesions were observed in 16-33% of patients. Dose escalation of TNF-inhibitors was often required to maintain remission (*29*). TNF-inhibitors control TA without curing the disease: disease relapsed in 13 of the overall 14 patients with suspended biologic treatment after longstanding-remission (*24-26*). A satisfactory response was observed after resuming TNF-blockers.

Recently, interleukin-6 (IL-6) has been assessed as a therapeutic target for TA. IL-6 is a pleiotropic cytokine with local and systemic actions. IL-6 influences the function of many cell types present in TA arterial lesions: IL-6 is important for B- and T-lymphocyte differentiation, generation of Th17 cells, fibroblast proliferation and hepatic synthesis of acute-phase proteins, including C-reactive protein (CRP) (*30*). IL-6 is expressed in TA aortic lesions and serum IL-6 levels are elevated in TA, particularly during active phases (*23,31,32*). Very preliminary experienceproposed that tocilizumab, a humanized anti-IL6 receptor antibody, could be another option for refractory TA (*33-38*). Very recently, a meta-analysis observed a steroid-sparing activity of tocilizumab with a good clinical and laboratory response (*39*). Even if arterial 18F-fluorodeoxyglucose (^{18}F-FDG) uptake at PET examination was reduced after tocilizumab therapy, radiological activity did not apparently significantly decrease (*39*). Tocilizumab was effective in several patients refractory to TNF-inhibitors and relapses were frequent after its discontinuation, needing long-term maintenance therapy. Major limitations in this work are the heterogeneity in radiological follow-up between different studies and a median follow-up of tocilizumab after nine months, which is quite short considering that TA frequently has a chronic-relapsing course.

After this meta-analysis, three unicentric cohorts of TA patients treated with tocilizumab were published: Goel *et al*. reported that six of ten patients treated with tocilizumab for six months remained radiologically stable as evaluated by angiography or Doppler ultrasonography (*40*). Nekaoka *et al*. reported four patients (two of which previously received only steroids) on tocilizumab therapy for more than two years followed with both Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) (*41*). They observed imaging stabilization in two patients and improvement in the other two. Interestingly, serum IL-6 levels initially increased and then gradually were reduced concordantly with improvement of arterial thickened lesions, suggesting progressive reduction of IL-6 production.

We recently reported our experience with seven refractory patients treated with tocilizumab for a median of 14 months (*42*). All patients were followed with regular high-resolution ultrasonography and MRI. Each arterial lesion was assessed individually at radiological follow-up. Three out of our seven patients had a complete response and imaging evidence of stabilization or improvement of all arterial lesions. However, we had big difficulties in assessing TA activity, because tocilizumab causes normalization of acute-phase markers, such as CRP and erythrocyte sedimentation velocity (ESR) and resolution of systemic inflammatory symptoms (fever and malaise). This might not necessarily reflect an effective action of the agent on the actual pathway responsible for vessel progression, but only interference with the systemic pleiotropic function of the cytokine.

This implies that inflammatory reactants might be biased when assessing activity during tocilizumab therapy. Similarly, the accuracy of disease activity indices, such as the National Institutes of Health (NIH) criteria or the Indian Takayasu Activity Score (ITAS) may be compromised. Regular morphologic vascular imaging with MRI, CT and Doppler ultrasonography is thus fundamental for assessment of response and

TA activity using tocilizumab (*42,43*). If functional imaging (and ^{18}F-FDG-PET in particular) can overcome this problem and allow identification of progression is still an open issue. Similarly, it is unknown if therapy with tocilizumab or other biological agents influences the accuracy of functional imaging. We feel that these points should receive greater attention before relying on functional imaging for clinical decisions.

Our study also raises questions about the mechanism of vascular progression, which we observed in 4/7 patients despite adequate therapy targeting the pivotal proinflammatory pathway of IL-6 and in the absence of systemic inflammatory reaction. Probably other local inflammatory pathways cooperate in the process. Locally-produced inflammatory molecules whose generation is independent of IL-6, such as pentraxin-3 (a long pentraxin produced directly within the sites of arterial inflammation), have indeed been shown to detect TA activity other than ESR and CRP (*44*). In addition to secreted molecules such as cytokines, also infiltrating leukocytes and resident cells likely represent important factors of this local inflammatory response. This cellular component may represent promising therapeutic targets for TA. Examples of this strategy are the anti-CD20 antibody rituximab and the fusion molecule abatacept. However, experience with these agents for refractory TA are still very embryonic (*19*) and a randomized controlled trial with abatacept for large-vessel vasculitis is currently recruiting patients (*45*).

Inflammation and damage perturb tissue homeostasis evoking responses that may lead to tissue remodelling. As such, local reparative responses are likely activated at the sites of arterial lesions. Alterations in blood flow and shear stress may further modulate arterial remodelling. Currently, arterial wall response to inflammation, damage and alteration of shear stress in TA has been a neglected issue.

Peculiar tissue remodelling responses have been advocated in the observation that operative (endovascular or surgical) therapies have worse long-term patency rates in TA than in occlusive arteriosclerotic disease (*19*). This constitutes the major pitfall of operative therapies in TA. Current (scarce) evidence reports that myointimal hyperplasia is the main cause of restenosis and long-term failure. Structural characteristics of TA lesions in comparison to atherosclerosis, such as longer length and lower arterial wall compliance, may result in suboptimal dilatation and in more mechanical damage due to higher inflating pressures and may partly explain the observed worse long-term results (*19*). However, the TA inflammatory milieu probably influences post-interventional arterial wall remodelling (*8,46-49*), because a lower restenosis rate is observed when vascular interventions are carried out during remission (*18,50*) and when post-interventional immunosuppressive therapy was added onto steroid regimens used to control disease activity (*50*).

These observations suggest that local arterial inflammation and arterial wall remodelling may cooperate for vascular progression and account at least partially for the inaccuracy of the systemic inflammatory markers in addressing TA activity and in the heterogeneity of response to biologic agents that block pivotal proinflammatory pathways such as TNF and IL-6. Further research is needed to clarify the pathogenesis of this still orphan and mysterious disease.

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

Example 4 (Sample web page reference):
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