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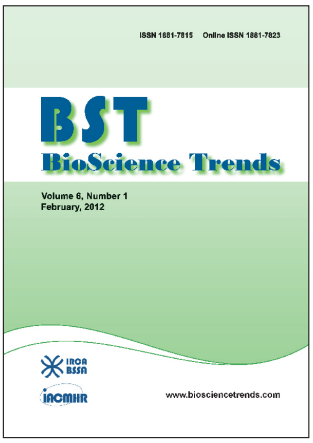

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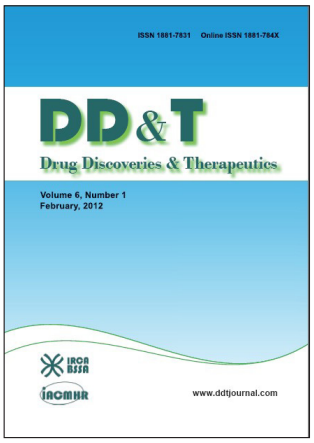

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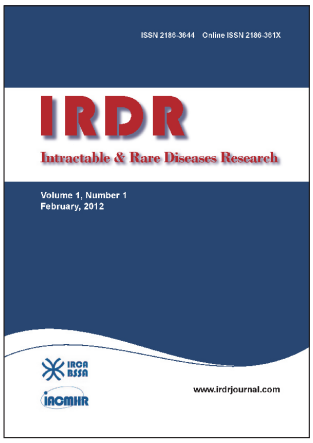

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Mini-Review

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The role of L-type amino acid transporter 1 in human tumors

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Summary **L-type amino acid transporter 1 (LAT1) is an L-type amino acid transporter and transports large neutral amino acids such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine, and histidine. LAT1 was found to be highly expressed especially in human cancer tissues, and up-regulated LAT1 can lead to dysfunction in human tumor cells. These findings suggest that LAT1 plays an important role in human tumors. This review provides an overview of the current understanding of LAT1 expression and its clinical significance and function in tumors.**

Keywords: LAT1, human tumor, proliferation, angiogenesis

1. Introduction

Cancer cells require a large amount of nutrients and amino acids for rapid growth and continuous proliferation. This situation is facilitated by the upregulation of amino acid transporters. Amino acid transporters located on the plasma membrane facilitate the movement of amino acids cross the cytoplasm. System L is a major transport system providing cells with large neutral amino acids, including branched or aromatic amino acids (*1*). To date, four L-type amino acid transporters (LATs), LAT1-LAT4, have been identified at the molecular level. LAT1 has been found in many malignant cells, while LAT2 functions in the epithelium of the kidney proximal tubules and digestive tract. LAT3 and LAT4 have been localized to the apical plasma membrane of podocytes and to the distal tubules and collecting ducts (*2*).

Among the known LATs, LAT1 has garnered particular attention because of its limited distribution and higher expression in malignant tumors. Previous studies have demonstrated that LAT1 is regulated in various tumors to increase amino acid transport (*3*). LAT1 can transport large neutral amino acids such leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine, and histidine (*4-6*). Encoded by *SLC7A5*, the 55-kD protein forms 12 putative transmembrane domains (*7*), and the functional expression of LAT1 requires covalent association of the heavy chain of 4F2 cell surface antigen (CD98) (*8*). LAT1 is also an exchanger, and it can exchange intracellular glutamine for external large neutral amino acids. The apparent affinity for large neutral and aromatic amino acids is in the physiological micromolar range on the extracellular portion and up to 100-fold higher on the cytosolic portion of the transporter (*9*).

Because of its proposed role in supplying nutrients necessary for tumor growth and proliferation, LAT1 may be a critical target for cancer intervention. The current review provides an overview of the current understanding of the clinical significance of LAT1 expression and its function in tumors.

2. Expression of LAT1 in various tumors

Although LAT1 can provide essential amino acids for normal cell growth, its expression is limited to organs such as the brain, spleen, thymus, and testes. Importantly, however, LAT1 is also highly expressed in human cancer tissues (*10,11*), including cholangiocarcinoma, multiple myeloma, malignant glioma, and lung, uterine cervical, oral, prostate, and breast cancer. The use of molecular techniques has revealed that the level of LAT1 expression in malignant tumor tissues is significantly higher than that in surrounding healthy tissues and benign tumor tissues. Moreover, the expression of LAT1

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in malignant tumor tissues with distant metastasis is higher than that in tissues without distant metastasis., LAT1 is over-expressed in human gliomas and is predominantly expressed in the vascular endothelium and the cytoplasm of tumor cells, as well as in the plasma membrane of tumor cells (*12*). In addition, the level of LAT1 expression is higher in infiltrating glioma cells than in cells located in the center of a tumor (*13*). These findings suggest that LAT1 may be associated with the metastasis of tumors in humans. Similar results were reported for uterine cervical carcinoma, in which LAT1 expression is limited to the basal layer of normal squamous epithelium, and the level of LAT1 expression in invasive squamous cell carcinoma is significantly higher than that in cervical intraepithelial neoplasia (*14*). In the lungs, LAT1 is not detected in normal epithelial cells but its expression is noted in non-small lung cancer; in addition, LAT1 expression is significantly higher in patients with mediastinal lymph node metastases than in patients without those metastases (*8*). Interestingly, non-solid tumors also display altered LAT1 expression: LAT1 acts an activation antigen in T lymphocytes and T-cell leukemia results in higher levels of LAT1 expression compared to normal activated T cells (*15*). The distribution, level of expression, and methods of detection of LAT1 are summarized in Table 1.

Thus, a wealth of evidence indicates that the level of LAT1 expression is abnormally high in human cancer cells. However, the mechanism underlying this expression remains unclear. Yamauchi *et al*. reported that LAT1 can activate the mammalian target of the rapamycin (mTOR) signaling pathway, which plays an important role in protein synthesis and energy supply (*16*). However, few studies have identified the molecular mechanisms by which LAT1 may be promoting tumorigenesis.

3. The biological activity of LAT1 in tumors

Since LAT1 is overexpressed in various types of tumors, the question of whether up-regulation of LAT1 leads to the transformation of human tumor cells, or whether its overexpression is a by-product of tumorigenesis, must be considered. Previous studies have reported that LAT1 can regulate multiple biological processes, including cell growth, invasion, and angiogenesis, that primarily characterize malignant tumors.

3.1. *LAT1 and tumor cell proliferation*

Amino acids are essential for protein synthesis, which is necessary for tumor cell growth. LAT1 can mediate amino acid uptake, and its upregulation in tumor cells suggests that LAT1 may promote tumor cell growth. Importantly, many studies have demonstrated that inhibition of LAT1 can reduce tumor cell proliferation.

Apoptosis, the major form of cell death, is deregulated in tumors, allowing their continuous growth. The upregulation of LAT1 in tumor cells may affect caspase activity, thereby altering apoptosis. Kim *et al*. reported that in KB, Saos2, and C6 cell lines, down regulation of LAT1 by 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) inhibits cell growth by activating apoptosis through the induction of caspase-3 and caspase-7 (*17*). Similarly, Kobayashi *et al*. reported that over-expression of LAT1 in gliomas with low endogenous expression of LAT1 significantly enhanced the rates of tumor cell growth in athymic mice but that treatment with BCH promoted apoptosis through the activation of caspases (*18*).

3.2. *LAT1 and tumor cell invasion*

Tumor invasion and metastasis are the major causes of morbidity and death in cancer patients. The supply of nutrients, and especially amino acids, is critical to this process. LAT1 upregulation is associated with tumor cell invasion, and down-regulated LAT1 can suppress tumor cell invasion. Indeed, cell migration and invasion were reduced after LAT1 knockdown in cholangiocarcinoma cells (*3*). Similarly, downregulation of LAT1 expression can inhibit the invasion and migration of gastric cancer cells (*19*). However, the exact mechanism underlying this process is not fully understood.

3.3. *LAT1 and tumor angiogenesis*

Angiogenesis is critical to tumorigenesis because new blood vessels are necessary to supply nutrients and oxygen and to dispose of metabolic waste products. Moreover, an enhanced vascular supply could reflect malignant potential. Many studies have demonstrated that LAT1 is associated with angiogenesis. Indeed, the protein is observed in vascular endothelium, and its level of expression is markedly associated with glioma microvessel density (*12*). Moreover, the expression of LAT1 is correlated with tumor angiogenesis as assessed by vascular endothelial growth factor expression, microvessel density, and vascular invasiveness of tumors (*8*).

4. Clinical significance of LAT1 in tumors

Since LAT1 functions as an amino acid transporter, its clinical significance in cancer can be traced to differences in amino acid transport within tumors. Several studies have noted an increased uptake of radio-labelled amino acids, including 6-18F-fluoro-L-3,4-dihydroxy-phenylalanine (^{18}F-DOPA), L-[3-^{18}F]-α-methyl tyrosine (^{18}F-FAMT), and anti-1-amino-3-[^{18}F]fluorocyclobutane-1-carboxylic acid (anti-[^{18}F] FACBC), in human cancers (*20*). The uptake of these radio-labelled amino acids is mediated by LAT1, with a direct correlation between uptake levels and levels

Table 1. Summary of reported distribution, level of expression, and methods of detection of LAT1 in human tumors

Link to disease	Expression	Method of detection	*Ref.*
Uterine cervical carcinoma	Higher in invasive squamous cell carcinoma than in cervical intraepithelial neoplasia	Immunohistochemistry	(*14,34*)
Non-small cell lung cancer	Higher in patients with mediastinal lymph node metastases than in those without	Immunohistochemistry, quantitative real-time PCR	(*4,27*)
Oral cancer	High	Immunohistochemistry	(*22,35,36*)
Breast cancer	High	Immunohistochemistry, quantitative real time PCR, Western blotting	(*37,38*)
Renal cell carcinoma	High	Quantitative real-time PCR	(*20*)
Esophageal squamous cell carcinoma	High	Immunohistochemistry	(*37*)
Leukemic	High	Western blotting	(*15*)
Cholangiocarcinoma	High	Quantitative real- time PCR	(*4*)
Multiple myeloma	High, associated with increased proliferation	Immunohistochemistry	(*39*)
Malignant gliomas	Higher in infiltrating glioma cells than in cells located in the center of the tumor	Immunohistochemistry	(*13*)
Gastric cancer	High	Quantitative real-time PCR, Western blotting	(*40*)
Prostate cancer	High	Immunohistochemistry	(*28,31,41*)
Thymic carcinomas	High	Immunohistochemistry	(*18*)

of LAT1 expression. In particular, LAT1 expression is significantly correlated with L-3,4-dihydroxy-(ring-2,5,6-3H) phenylalanine (^{3}H-*L*-DOPA) uptake in human gliomas *in vitro* and 18F-DOPA uptake *in vivo* (*21*). Similar, in oral squamous cell carcinoma the uptake of ^{18}F-FAMT is mediated by LAT1 expression. Moreover, ^{18}F-FAMT positron emission tomography (PET) imaging has displayed a higher specificity at detecting malignant lesions than 2-[^{18}F]fluoro-2-deoxy-D-glucose (^{18}F-FDG) PET (*22*). In breast cancer, over-expression of LAT1 is correlated with anti-[^{18}F] FACBC, which can serve as a potential biomarker for diagnosis of breast cancer (*23*). Thus, evidence has demonstrated that the relationship between radio-labelled amino acids and LAT1 expression can be used to diagnose cancer.

LAT1 may also be a promising molecular target for human cancer therapy. BCH, as an inhibitor of the system L amino acid transporters, suppresses cancer cell growth and migration. Specifically, inhibition of LAT1 has significant anti-tumor action on cholangiocarcinoma and augments the therapeutic efficacy of 5-fluorouracil (5-FU) and gemcitabine (GEM) (*4*). Inhibition of LAT1 by BCH also has anti-tumor action in non-small cell lung cancer. Moreover, BCH reduced mortality in a model involving C6 glioma-bearing rats (*24*). Importantly, though, BCH is not a highly specific inhibitor of LAT1. In contrast, JPH203, a novel tyrosine analog, has a high level of selectivity for LAT1. Administration of JPH203 can effectively induce suppression of cell growth and cell apoptosis in YD-38 human oral cancer cells (*25*) and also inhibit cell growth in human colon and leukemia cancer cells (*15,26*). The knockdown of human LAT1 by small interfering RNAs or stable transduction with lentivirus can also lead to the inhibition of cancer cell growth and migration (*27*). Similarly, down- regulating LAT1 can lead to decreased growth of prostate cancer cells (*28*) and human oral cancer cells (*29*). However, the over-expression of LAT1 has been suggested as a target for combination therapy with anti-proliferative aminopeptidase inhibitors to combat ovarian cancer (*30*). Recent studies have proposed that LAT1 may be useful as a targeted drug transporter (*31*). Nonetheless, further work is needed to uncover its potential utility in clinical settings.

To date, surgical resection is still the primary treatment for human cancer, but the prognosis after treatment remains poor. Therefore, clinical markers that can predict the response to a specific therapy and aid in determining prognosis should be identified. LAT1 has been used as a prognostic marker in a variety of tumors types. For example, a high level of LAT1 expression is a significant factor for predicting a poor outcome after surgical resection. Specifically, the over-expression of LAT1 is a pathological factor for predicting the prognosis for patients with surgically resectable stage III non-small cell lung cancer (*8*). Patients with hepatocellular carcinoma and a high level of LAT1 expression are reported to have a significantly shorter overall survival (*5*). Similarly, in prostate cancers, over-

expression of LAT1 can predict local progression under expectant management (*32*). Over-expression of LAT1 can also serve as a novel independent biomarker of high-grade malignancy, which can be utilized together with the Gleason score, to assess prognosis (*33*). Thus, LAT1 may be useful as a prognostic marker to predict a poor outcome after surgical resection.

5. Conclusions and perspectives for the future

In conclusion, LAT1 plays a critical role in the formation and development of cancer, and a high level of its expression apparently has clinical significance. As LAT1 is studied, new opportunities are arising to determine the mechanisms of tumor origin and progression. Thus, the potential exists to prevent, diagnose, assess, and treat malignancies by intervening in LAT1 expression or activity. Although promising, further studies are needed to discover and optimize its therapeutic uses in the future.

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Review

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Sarcoidosis and the heart: A review of the literature

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Summary **Sarcoidosis is a chronic multisystem disorder without any defined etiology. Cardiac sarcoidosis (CS) is detected in 2-7% of patients with sarcoidosis and more than 20% of the cases of sarcoidosis are clinically silent. Cardiac involvement in systemic sarcoidosis (SS) and isolated cardiac sarcoidosis (iCS) are associated with arrhythmia and severe heart failure (HF) and have a poor prognosis. Early diagnosis of CS and prompt initiation of corticosteroid therapy with or without other immunosuppressants is crucial. Electrocardiography, Holter monitoring, and Doppler echocardiography with speckle tracking imaging can serve as the initial steps to diagnosis of CS. Cardiac magnetic resonance (CMR) imaging and positron emission tomography (PET) are promising techniques for both diagnosis and follow-up of CS. This review discusses the main aspects of cardiac involvement in sarcoidosis.**

Keywords: Sarcoidosis, cardiac involvement, diagnosis, treatment

1. Introduction

Sarcoidosis, formerly called Mortimer's Malady, is a chronic multisystem disorder without any defined etiology. It is characterized by noncaseating granulomas in the affected organs or tissues (*1*). Its incidence varies from 3-4 to 35-80 per 100,000 according to ethnicity, region, and gender (*2*). Lymph nodes and lungs are the most frequently affected tissues, but sarcoidosis can also affect other organs and tissues like the skin, the central nervous system, the eyes, muscle, bone, and the heart (*1,2*). Cardiac sarcoidosis (CS) is detected in 2-7% of the patients with sarcoidosis, but more than 20% of the cases of CS are clinically silent (*3*). Interestingly, cardiac involvement can be as high as 58% in Japanese patients with sarcoidosis and CS is responsible for 85% of the deaths due to sarcoidosis in this population (*1*). Complete heart block, bundle branch block, ventricular tachycardia (VT), congestive heart failure (HF), and sudden death are common presentations in CS (*1*). Endomyocardial biopsy (EMB), electrocardiogram (ECG), Holter monitoring, two-dimensional and Doppler echocardiography including strain imaging, radionuclide studies, cardiac magnetic resonance (CMR) imaging, and positron emission tomography (PET) are among the main techniques used to diagnosis CS. Corticosteroids with or without immunosuppressants are the mainstay of therapy for CS. This review will summarize the epidemiologic, pathophysiologic, diagnostic, clinical, and therapeutic aspects of CS.

2. Epidemiology

Sarcoidosis is a chronic multisystem disorder, characterized by noncaseating granulomas in multiple tissues and organs. According to previous data, sarcoidosis has a prevalence of 10-40/100,000 persons in the United States and Europe. Interestingly, African-Americans have a higher prevalence of the disease compared to Caucasians, with a ratio between 10 and 17 to 1 (*4*). Similarly, the Scandinavians have a higher prevalence of sarcoidosis than other whites (*5*). A study in Turkey found the incidence of sarcoidosis to be 4 per 100,000 (*6*). Sarcoidosis is said to have a slight sex preference since females between the ages of 20 and 40 have the highest incidence of systemic sarcoidosis (SS), but myocardial involvement does not show any gender preference according to the current data (*7,8*). CS can

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be part of SS or it can be detected in an isolated form. According to a pathology series, cardiac involvement occurs in 20-30% of patients with sarcoidosis (*6*). Cardiac involvement is associated with a poor prognosis (*9*). Myocardial granulomas were detected in 27% of 84 autopsies of patients with pulmonary sarcoidosis (PS) (*10*). In Japanese patients with sarcoidosis, cardiac involvement was reported to be high as 58% (*11,12*). Cardiac involvement in sarcoidosis can be responsible for up to 85% of the deaths among Japanese patients with sarcoidosis (*12,13*). In clinical practice, however, only 5% of patients with sarcoidosis have clinical manifestations of heart disease, and about 50-60% of patients with CS diagnosed at autopsy were not diagnosed with the disease while they were living (*1*). According to a study by the American Thoracic Society in 1999, respiratory failure is the most common cause of mortality among patients with sarcoidosis, accounting for an overall mortality of 1 to 5% (*8*). In contrast to previous studies, isolated cardiac sarcoidosis (iCS) is much more common than suspected (*3*). In a previous autopsy study, 40% of patients with CS had no signs of extracardiac involvement (*3,14*); in a retrospective study, 66% of patients with CS had disease isolated to the heart (*3*).

3. Pathogenesis and Etiologic Factors

The etiology and pathophysiology of sarcoidosis has not been fully understood, but the literature features some promising data that can help to understand the mechanism at the core of the disease process. Discrete, compact, noncaseating epithelioid cell granuloma is the principal lesion found in organs affected by sarcoidosis (*8*). These epithelioid cell granulomas consist of highly differentiated mononuclear phagocytes (epithelioid cells and giant cells) and lymphocytes (*15,16*). Granuloma formation occurs as a result of a cell-mediated delayed hypersensitivity immune reaction in individuals with immune dysfunction. After macrophages phagocytize the antigen, they present the antigen and effector CD4+helper T cells secrete IL-2 and IFN-γ that trigger a Th1 immune response. Non-necrotizing granuloma is formed as a result of the collection of highly differentiated mononuclear phagocytes (epithelioid cells and multinucleated giant cells), Schaumann bodies or asteroid bodies, patchy fibrosis, and lymphocytes (*3,15,16*). Three categories of potential etiologic factors have previously been defined: infective, noninfective, and genetic (*17*). Viruses (herpes, Epstein–Barr, retrovirus, coxsackie B virus, and cytomegalovirus), Borrelia burgdorferi, Propionibacterium acnes, Mycobacterium tuberculosis and other mycobacteria, Mycoplasma orale, beryllium, aluminum, zirconium, clay, talc, hairspray, pine tree pollen, peanut dust, mineral oil, and drugs (*e.g.* sulfonamide or methotrexate) can induce granuloma formation in genetically-predisposed individuals with abnormal immune responses (*8,18-22*). The variability of disease presentation (pattern of disease, severity, and prognosis) among different races and in individuals with specific HLA sub-types and the presence of some familial clusters indicate a genetic susceptibility for sarcoidosis (*5,23,24*). First-degree relatives of patients with sarcoidosis were found to have a relative risk of sarcoidosis five times that of control subjects (*1,25*). In a case-control etiologic study of sarcoidosis (ACCESS) a significantly elevated risk of sarcoidosis was observed among first- and second-degree relatives of patients with sarcoidosis compared to that in relatives of matching control subjects (*26*). HLA analyses of affected families showed that the mode of inheritance of the risk for sarcoidosis can be polygenic, most commonly including the class I HLA-A1 and -B8 and class II HLADR3 genotypes (*27-29*). Genetically predisposed individuals are likely to develop granulomas after exposure to antigens that trigger an exaggerated cellular immune response (*8*). The presence of HLA-DQB1*0601 and the allele TNFA2 in Japanese female patients with CS also indicates a genetic etiology (*23,24*).

4. Clinical Manifestations

Although the incidence of cardiac involvement is higher in autopsies, the clinical manifestations of cardiac involvement are seen in about 5% of patients with sarcoidosis (*1,8,30*). The extent and location of granulomas are the determinants of the clinical manifestations of sarcoidosis. There are three consecutive histological stages including edema, granulomatous infiltration, and fibrosis leading to postinflammatory scarring (*1*). Granulomatous inflammation can involve either the myocardium, endocardium, or pericardium (*10,16,31,32*). The myocardium is the portion of the heart most commonly affected by CS, but the pericardium and endocardium are usually involved as a result of the spread of myocardial inflammation (*3,10,32,33*). The free wall of the left ventricle, interventricular septum (IVS), papillary muscles, right ventricle (RV), and atria can be involved, though with less frequency (*3,14,32*). A physician should be alert for CS if there is fibrosis and scar formation in unusual myocardial regions atypical of coronary ischemia in the absence of coronary artery disease (CAD) in a young individual (*3*).

There is significant variability in clinical presentation ranging from benign arrhythmia to severe heart block and sudden death (*7,8*). The clinical manifestations also vary from patient to patient (*7*). The presence of mere cardiac symptoms such as palpitations should be carefully evaluated. In previous studies, the most common cardiac presentations were allocated into three major groups: arrhythmia, cardiomyopathy, and pericardial involvement (*1,7,12*). The prevalence

of arrhythmia ranges from 0 to 65%. The prevalence of specific arrhythmias is as follows: 26-62% in AV block, 12-61% in bundle branch block, 0-15% in supraventricular tachycardia, 2-42% in VT, and 12-65% in sudden cardiac death (*7*). In patients with CS, complete heart block is among the most common arrhythmias and occurs in younger patients in contrast to older patients presenting with complete heart block due to other causes (*34*). Scarring or granuloma formation in the basal septum or involvement of the nodal artery leading to ischemia in the conduction system can result in complete heart block and bundle branch block (*12*). Complete heart block can directly cause sudden cardiac death. Interestingly, Japanese women over 50 years of age are frequently admitted with complete heart block, leading to diagnosis of CS in 11% of cases (*35*). VT is another common tachyarrhythmia in CS (*7*). In a previous study by Sekiguchi *et al*., sudden cardiac death due to ventricular tachyarrhythmia and complete heart block was reported to cause 25-65% of the deaths due to CS, and the study also indicated that sudden death can be the initial presentation in 40% of patients with CS (*36*). Abnormal automaticity, reentrant circuits due to sarcoid granulomas, or scar tissue can lead VT (*1*). In an emergency setting, CS should be considered in cases of sudden cardiac death with no definite etiology. Atrial arrhythmia is less common than ventricular arrhythmia and often results from atrial dilatation or pulmonary involvement rather than atrial granulomas (*32*).

Cardiomyopathy was reported to have a prevalence of 10-30% (*1,7,12*). Left ventricular (LV) systolic failure, HF with preserved ejection fraction, or right ventricular failure secondary to pulmonary disease are the main manifestations of cardiomyopathy in sarcoidosis (*1,7,12*). According to one study, 25% to 75% of cardiac deaths in patients with CS are due to progressive HF (*33*). CS can be difficult to differentiate from idiopathic dilated cardiomyopathy (IDC) (*1*). A significantly higher frequency of complete heart block (67% *vs*. 0%), right bundle branch block (57% *vs*. 17%), and abnormal left ventricular wall thickness (73% *vs*. 17%) in sarcoidosis can help to exclude IDC (*33*).

Pulmonary hypertension (PH), a predictor of poor prognosis, was found to have a prevalence of 73.8% in advanced sarcoidosis (*37*). In a previous study at a Japanese outpatient clinic, PH was found to be present in 5.7% of cases of CS (*38*). PH can be due to impaired forward flow because of poor left ventricular function and can result from PS in patients with hypoxic vasoconstriction leading to cor pulmonale (*1*). PH can be caused by encroachment of the pulmonary vasculature due to intimal and medial infiltration by noncaseating granuloma and extrinsic compression of pulmonary arteries by enlarged mediastinal lymph nodes (*39*). PH is diagnosed based on an estimation of right ventricular systolic pressure (RVSP) using Doppler echocardiography and a modified Bernoulli equation. RVSP is considered to be equal to the systolic pulmonary artery pressure (sPAP) in the absence of right ventricular outflow obstruction. It is calculated as follows: sPAP = right ventricular systolic pressure = transtricuspid gradient + right atrial pressure, where the transtricuspid gradient is $4v^2$ (v = peak velocity of tricuspid regurgitation in meters per second) (*40*). According to the WHO criteria for classification of PH, sarcoidosis is included in group 5, which includes PH with unclear multifactorial mechanisms (*41*).

Pericardial involvement is detected in 20% of patients with CS. Pericardial involvement is most commonly evident as pericardial effusion detected in echocardiography. Pericarditis is a rare clinical presentation (*1,7,12*). Direct granulomatous involvement of cardiac valves (less than 3%), coronary artery granulomatous disease leading to myocardial ischemia, constrictive pericarditis, and intracardiac masses are other rare clinical presentations of CS (*1,7,42-44*). Although direct valvular involvement is rare, valvular insufficiency secondary to papillary muscle dysfunction is seen in 68% of patients with CS (*42*).

Another issue in CS is ventricular aneurysms. These occur in 10% of patients with sarcoidosis (*1*). The most commonly affected areas are the anterior and septal walls, and apical involvement alone is very rare (*1*). Fibrotic tissue formation due to long-term corticosteroid use to treat cardiac granulomas and extension of myocardial sarcoid lesions can lead aneurysm formation (*45,46*). However, patients with untreated CS can develop myocardial aneurysms, so corticosteroids should be used if indicated (*1*). Frequent and complex ventricular arrhythmias can be seen in patients with myocardial aneurysms (*1*). Since impaired arterial perfusion in the proximity of cardiac granulomas can impair the local delivery of antiarrhythmic drugs and certain acidic acute phase molecules can react with antiarrhythmic drugs with a high pK to reduce their serum levels, resection of the aneurysm can be an option for treatment of intractable ventricular tachyarrhythmia (*1*).

5. Diagnosis

The diagnosis of cardiac involvement in sarcoidosis is somewhat challenging (*2*). There were no clinical signs or symptoms of the disease in 37% of patients with cardiac involvement (*1*). Early diagnosis and prompt initiation of antiinflammatory therapy is crucial to preventing poor outcomes (*1,47*). Nevertheless, there is no gold standard to test for CS (*2*). Over the past ten years, some important diagnostic and management strategies have been proposed like the revised Japanese Ministry of Health and Welfare Guidelines (JMHWG) from the Japan Society of Sarcoidosis and Other Granulomatous Disorders and the Delphi study (*48,49*). However, there is lack of consensus regarding

the management of CS (*2*). Medical history, physical examination, ECG, 24-hour Holter monitoring, and echocardiography should be the components of initial clinical evaluation (*2*). Patients may have some nonspecific symptoms like chest pain, palpitations, syncope, bradycardia, peripheral edema, dyspnea, and orthopnea (*50,51*). In a previous study examining a cohort with CS, patients presented with atrioventricular block (50%), left-sided HF (40%), syncope (31%), palpitations (17%), chest pain (14%), and bradycardia (10%) (Table 1) (*50*). Clinical findings can be helpful in drawing conclusions about the extent of disease and inflammatory activity (*2*). A previous prospective study reported that at least one abnormal screening result, including cardiac symptoms, a cardiac examination, 12-lead ECG, echocardiogram, and Holter monitor, had a 100% sensitivity and 87% specificity at detecting CS, with history/examination, an echocardiogram, and Holter monitor being the most useful (*47*).

5.1. *Electrocardiography*

A resting ECG is commonly accepted as an appropriate test to screen for patients with sarcoidosis (*3,8,47,52*). ECG was reported to have a sensitivity of 33% to 58% and a specificity of 22% to 71% at detecting CS (*53,54*). ECG abnormalities like conduction disturbances, arrhythmia, or nonspecific ST and T-wave changes have been detected in 20 to 31% of patients with sarcoidosis (*10,55-57*). An autopsy study of sarcoidosis with mild (microscopically evident granulomas) and severe (gross evidence of cardiac granulomas or infiltration at autopsy) cardiac involvement reported finding arrhythmia in 42% of patients and conduction disturbances in 75% of patients (*10*). ECG can be useful in estimating the extent of disease or inflammatory activity but only persistent ventricular tachycardia can predict an adverse outcome (*58*). Although the role of signal-averaged ECG (SAECG) in diagnosing CS is unclear, a recent study reported that it had a sensitivity of 52% and specificity of 82% as a technique to screen for CS (*59*). Holter monitoring can be a predictor of cardiac involvement in sarcoidosis with a sensitivity of 50% and a specificity of 97% when using CMR or PET as a reference (*47*). Another study concluded that Holter monitoring is a powerful screening tool with which to predict a positive CMR or PET scan (*60*). Yet another study reported that 24-Holter monitoring had a sensitivity of 67% and a specificity of 80% at detecting CS (*61*).

Table 1. Common presentations of patients with CS

Atrioventricular block	50 %
Left-sided heart failure	40 %
Syncope	31 %
Palpitations	17 %
Chest pain	14 %
Bradycardia	10 %

5.2. *Echocardiography*

Echocardiography is another important tool with which to diagnose CS. Echocardiographic abnormalities are detected in 24-77% of patients with CS (*7,62-64*). These abnormalities include abnormal septal thickening or thinning, dilatation and systolic dysfunction of the LV, regional wall motion abnormalities without involvement of the coronary arteries, a focal intracardiac mass caused by a large granuloma, diastolic dysfunction, valvular regurgitation, papillary muscle dysfunction, pericardial effusions, and macroscopic areas of bright echoes indicating granulomatous inflammation (a speckled or snowstorm pattern) (*1,3,7,31,33,42,65-67*). Further investigation is necessary if a patient has extracardiac sarcoidosis with abnormal 2-D echocardiographic findings and subtle abnormalities in diastolic flow patterns (*7*). A previous retrospective study reported that Doppler echocardiography was abnormal in 67% of patients, with abnormalities that included dilated cardiomyopathy (32%), abnormal left ventricular relaxation (29%), and diffuse or localized dyskinesia or hypokinesia (26%) (*1,53*). A previous study reported that 14% of patients with pulmonary sarcoidosis without known cardiac involvement had diastolic dysfunction as a result of CS (*68*). A prolonged isovolumic relaxation time and a reversed E/A Doppler ratio are the most common echocardiographic patterns of diastolic dysfunction seen in early CS (*68*). Although these Doppler findings have some role in diagnosing CS and determining its prognosis, they lack the sensitivity and specificity to detect early cardiac involvement (*7*). The cycle-dependent variation of myocardial integrated backscatter may involve mechanisms such as decreased regional myocardial contraction, altered myocardial acoustic properties due to myocytolysis, and cell infiltration in the myocardium; this variation may be reduced in the basal septum even in the absence of 2-D echocardiographic abnormalities, providing a new technique for detection of cardiac involvement (*1,69*). In a recent clinical prospective cohort study by Degirmenci *et al.*, the role of speckle tracking echocardiography (STE) was evaluated in patients with PS without clinical or echocardiographic evidence of cardiac involvement (*70*). The left atrial global longitudinal strain (LAGLS), total atrial conduction time (TACT), and LV function were studied in patients with PS (*70*). The results were as follows: LAGLS was significantly lower, TACT was significantly longer, LV longitudinal strain and strain rate (SR) measurements were significantly lower, and LVR-apical and LV-torsion (LVTR) values were significantly higher in patients with recently diagnosed sarcoidosis than in healthy controls (*70*). Thus, identification of left atrial and LV myocardial deformations with speckle tracking echocardiography can indicate subclinical LV dysfunction and subclinical electrophysiologic changes in patients with PS and aid

the physician in prompt initiation of therapy (*70*).

5.3. *Cardiac Magnetic Resonance Imaging/Positron Emission Tomography/Radionuclide Scintigraphy*

CMR imaging with a high spatial and soft-tissue resolution detects the active, inflammatory phase of disease and the chronic phase that includes mostly scarring and fibrosis in both SS and iCS (*2*). Focal wall thickening due to infiltration or edema and wall motion abnormalities seen on T1-weighted (cine) images, increased signal intensity on T2-weighted images, and early gadolinium enhancement are characteristics of the inflammatory phase (*11*). Wall thinning and delayed gadolinium enhancement, indicating myocardial damage, scarring, and fibrosis are findings in the chronic phase (*71*). Delayed gadolinium enhancement was recently reported to be the strongest hallmark of CS (*49*) and was reported to be associated with adverse events and cardiac death (2). Gadolinium enhancement can be useful in evaluating the response to steroid therapy (*72,73*). CMR imaging is probably more sensitive than radionuclide imaging (*11,51*) and has a similar sensitivity and a highly improved specificity in detecting CS compared to PET (*74,75*).

PET with 18F- fluorodeoxyglucose (FDG) is a form of functional imaging that indicates inflammation and that is useful in early diagnosis, monitoring of therapy, and image-guided biopsy (*76*). A patchy, focal uptake pattern specifically indicates CS (*2,3*). There are several ways in which 18F-FDG uptake is characterized (*77*), including no uptake, diffuse uptake, focal uptake, and focal on diffuse uptake (*78*). Other researchers have characterized patterns while incorporating data from perfusion and 18F-FDG PET images: normal perfusion and normal 18F-FDG, either abnormal perfusion or abnormal 18F-FDG, or both abnormal perfusion and abnormal 18F-FDG (*79*).

The degree of abnormal perfusion and 18F-FDG uptake can also be characterized as: normal (normal perfusion/normal 18F-FDG), early stage (mild perfusion defect/increased 18F-FDG), progressive stage (moderate perfusion defect/increased 18F-FDG), progressive myocardial impairment stage (severe perfusion defect/increased 18F-FDG), and fibrosis stage (severe perfusion defect/minimal or no 18F-FDG uptake) (*80*). These stages can be helpful in initial diagnosis and follow-up of patients and assessment of the response to therapy (*77*).

Combining 18F-FDG PET with a perfusion scan and ECG gating can rule out CAD and show resting perfusion defects due to inflammation-induced tissue damage (*76*). Cardiac imaging can be combined with whole-body imaging to evaluate extracardiac sarcoidosis lesions (*2*). In a previous meta-analysis, 18F-FDG PET imaging was reported to have a sensitivity of 89% and a specificity of 78% at detecting CS compared to the JMHWG (*81*). CMR is more specific at detecting scar formation in later stages of the disease process, but PET is more sensitive at detecting early stages of inflammation (*74*). As a result, combining PET and CMR can provide complementary data for the diagnosis of CS (*74*). In a previous study, 18FDG uptake on PET and focal perfusion detection were reported to have some impact on prognosis, including death and VT, in comparison to the Japanese criteria (*79*). Nevertheless, 18F-FDG-PET has some limitations, including physiological uptake of 18FDG in the myocardium in healthy subjects, physiologic uptake in normal myocardium on the basal and lateral LV walls, increased uptake in RV and IVS in PH because of the mechanical overload, and nonspecific uptake in non-sarcoid dilated cardiomyopathies (*82*).

Before the introduction of PET, 201 Tl, 99mTc-sestamibi, and 67 Ga scintigraphy were commonly used to diagnose and monitor cardiac involvement in sarcoidosis (*83*). Thallium-201 (201Th) or technetium-99 m (99mTc) resting perfusion scintigraphy can show areas of decreased uptake in CS due to fibrogranulomatous replacement, regional metabolic abnormalities, or microvascular vasoconstriction (*51,83-85*). In CS, perfusion defects commonly decrease with exercise and vasodilator infusion (reverse perfusion) (*54*). Accumulation of gallium-67 (67Ga) in areas of active inflammation allows the detection of CS (*7*). Unfortunately, 67Ga does not accumulate in areas of fibrogranulomatous scarring, so 67Ga scintigraphy has a lower level of sensitivity than other radionuclides (18-50%) (*11,51*). Recently, CMR and PET have replaced radionuclide studies in the detection of CS because of their superior attributes (*2,3,7*).

5.4. *Serum Markers*

There are no disease-specific markers for diagnosis of CS (*22*). Although serum angiotensin-converting enzyme (ACE) is elevated in 60% of patients with SS (*86,87*), it is not a sensitive marker and is detected in only 21.8% of patients with CS (*3,54,88*). Serum IgG (*89*), lysozyme (*90*), high-sensitivity troponin T (*90*), atrial and brain natriuretic peptides (*91*), and soluble IL-2 receptor (*89,92,93*) have been proposed as biomarkers, but they lack the sensitivity and specificity to detect CS or there are insufficient data regarding their role in CS (*22*).

5.5. *Endomyocardial Biopsy*

CS can be definitively diagnosed via an endomyocardial biopsy (EMB) indicating noncaseating epitheloid granulomas (*1*). However, the pitfalls of EMB are a low level of sensitivity (19-32%) and sampling and technical errors (*36,65,94,95*). Biopsies are commonly performed in the right ventricle, but they can be performed in the

left ventricle (*22*). EMB can reveal some nonspecific findings like myocardial interstitial fibrosis, myofibril disarrangement and fragmentation, and inflammatory mononuclear cell infiltrates (*16,36,96*). The free wall of the right ventricle and apical interventricular septum are the most common locations where biopsy specimens are obtained, but sarcoid granulomas are mostly located in free wall of the left ventricle or the basal septum (*3*). Because of the pathology and nonuniformity of sarcoid granulomas, those granulomas are seldom revealed by EMB (*94-96*). However, repeated and imaging-guided biopsies of the myocardium or mediastinal lymph nodes via CMR imaging or 18FDG-PET can be helpful and may improve the rate at which CS is detected (*94*). Since a biopsy is potentially fatal and imaging studies such as CMR imaging and PET are preferable options, EMB cannot be recommended as a routine tool for diagnosis of CS (*3,83,97,98*). Even if an EMB is unhelpful, cardiac involvement should be assumed in cases of sarcoidosis along with cardiac dysfunction and ECG abnormalities without any alternative etiology (*3*).

5.6. *Coronary Angiography*

Coronary angiography is commonly performed in patients with suspected CS in order to exclude CAD (*3*). Any wall motion abnormality can be detected during ventriculography and coronary arteries are typically normal (*3,99*). Vascular filling defects due to granulomatous vasculitis are rarely seen (*100*).

5.7. *Differential Diagnosis*

Dilated cardiomyopathy of any cause, arrhythmogenic right ventricular cardiomyopathy, idiopathic giant cell myocarditis, lymphocytic myocarditis, connective tissue diseases, vasculitis (Takayasu arteritis and Wegener granulomatosis), amyloidosis, dengue fever, Chagas disease, and other infectious causes like rheumatic fever, syphilis, fungal infections, and tuberculosis should be considered in the differential diagnosis of CS (*33,50,82,101-111*).

6. Prognosis, Therapy, and Follow-up

6.1. *Prognosis*

Cardiac involvement in SS and iCS is associated with arrhythmia and severe HF and has a poor prognosis (*22*). However, sarcoidosis without cardiac involvement is a relatively benign condition, and 28-70% of patients recover and most of their lesions disappear spontaneously within two years (*112,113*). The increased risk of sudden death in CS necessitates prompt initiation of antiinflammatory therapy (*1*). Recognizing lethal ventricular arrhythmia, including sustained VT and ventricular fibrillation, and ICD implantation for secondary prophylaxis are crucial to improving prognosis (*114*). If patients have or are likely to have CS according to different imaging modalities, a positive EMB is not necessary and medical treatment should be started immediately (*1*).

6.2. *Drug Therapy*

Corticosteroids are the mainstay of the initial therapy for CS (*1,22*). Long-term corticosteroid use was shown to be beneficial to patients with an LV ejection fraction (LVEF)> 55% and <54% by preventing LV remodelling and reducing the LV volume and increasing the LVEF (*115*). The same study also found that there was no beneficial effect of therapy in patients with an LVEF < 30%, highlighting the importance of the prompt initiation of therapy in the early or middle stages of the disease. Although there are scant data indicating that corticosteroid treatment improves prognosis, a previous study found that steroid therapy may improve survival, especially in patients with an LVEF > 50 % (*58,115,116*). Steroid therapy can alleviate an atrioventricular conduction disturbance (*35,117*) and reduce the frequency of premature ventricular beats and non-sustained VT (*118*). The evidence for use of other immunosuppressive drugs in CS, including methotrexate, azathioprine, leflunomide, mycophenolate mofetil, anti-TNFα antibodies, and hydroxychloroquine, is poor, but the use of these drugs may be reasonable in order to avoid long-term side-effects of corticosteroids, or these drugs can be given preference in cases where corticosteroids are contraindicated or the patient is resistant to corticosteroids (*114,119-124*). The optimal agents for the treatment of CS and the optimal duration of therapy remain to be elucidated (*3,22*). However, a treatment regimen including 3-day pulse intravenous methylprednisolone and prednisone 40 mg/day for a minimum of 4 weeks with a maintenance dose of 10 mg by 6 months may be reasonable (*3*). Dual or triple therapy with addition of azathioprine (or methotrexate or cyclophosphamide) and hydroxychloroquine, respectively, has been reported by Lynch *et al*. (*3*). During clinical relapses of CS, high-dose corticosteroids (IV pulse methylprednisolone) and/or immunosuppressive or cytotoxic agents may be required (*3*).

6.3. *Other Therapies*

ICD implantation is indicated for secondary prophylaxis in patients with lethal ventricular arrhythmia, including sustained VT and ventricular fibrillation (*114*). Antiarrhythmic drug therapy is controversial due to the high rate of recurrence and sudden death (*1*). Electrical ablation therapy may be efficacious in patients with sustained monomorphic VT despite medical therapy (*125-127*). Ventricular arrhythmia and heart block are among the key causes of morbidity and mortality in CS,

and appropriate risk stratification and implantable device considerations are required in all patients with CS (*7*). Although corticosteroid therapy can be efficacious at restoring AV conduction, implantation of a permanent pacemaker should be performed immediately in patients with a severe AV block (*1,7,118*).

Cardiac transplantation is reserved for end-stage disease unresponsive to medical therapy with angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, β blockers, and diuretics (*8,128*). Resistant ventricular tachyarrhythmia and severe intractable HF, especially in younger patients, are the major indications for cardiac transplantation (*1*). Starting corticosteroid treatment before the occurrence of severe systolic dysfunction can avoid cardiac transplantation (*1*). Sarcoidosis can develop in the transplanted heart 24 weeks to 19 months after transplantation (*1*).

6.4. *Follow-up*

Doppler echocardiography and STE at 3 months and PET and/or CMR imaging (at 3–6 months) can be used to follow up patients with CS (*3*). Serial PET/CT scans and an echocardiographic examination at 6-month intervals are reasonable for patients with complete remission (*3*). Using an ambulatory Holter ECG to observe for fatal arrhythmia should be considered for patients at 3 and 6 months (*129*).

7. Conclusion

Cardiac involvement in sarcoidosis is associated with a poor prognosis. The increased risk of sudden death in CS necessitates prompt initiation of antiinflammatory therapy. Medical history, physical examination, ECG, 24-hour Holter monitoring, and echocardiography should be the components of an initial clinical evaluation. This review has discussed 2D and Doppler echocardiography as well as a relatively new technique, STE. Using STE to identify left atrial and LV myocardial deformation can indicate subclinical LV dysfunction and subclinical electrophysiologic changes and aid the physician in the prompt initiation of therapy. The risk of sudden cardiac death in patients with CS necessitates regular monitoring by means of symptoms, ECG, ambulatory ECG, and echocardiography. The impact of CMR and PET imaging on diagnosis and follow up of CS and the smaller role played by EMB were also examined.

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Original Article

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Prediction of prognosis of ALS: Importance of active denervation findings of the cervical-upper limb area and trunk area

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Summary **Amyotrophic lateral sclerosis (ALS) is a motor neuron disease characterized by serious muscle atrophy and weakness. The purpose of this study was to find prognostic factors in patients with mild ALS using application forms for the Specified Disease Treatment Research Program in Japan. We classified ALS as mild, moderate and severe. The subjects consisted of 363 patients with mild ALS who underwent needle electromyography at registration and were followed for more than one year. Time to progression to severe ALS and time to deterioration of activities of daily living such as speech dysfunction, upper limb dysfunction, and walking disability were used as outcomes. Cox proportional hazards model analysis was performed to identify prognostic factors. Of the patients with initially mild ALS, 38.3% (139/363) had progressed severe ALS at the last follow-up. In multivariate analysis of time to progression to severe ALS, bulbar onset (hazard ratio [95% confidence interval]: 1.68 [1.13-2.49], p = 0.010), tongue atrophy (1.69 [1.14-2.51], p = 0.009), dyspnea (1.57 [1.02-2.41], p = 0.042) and active denervation findings (ADFs) of the cervical-upper limb area (1.81 [1.25-2.63], p = 0.002) emerged as prognostic factors. Furthermore ADFs in the trunk area were prognostic factors for upper limb dysfunction and walking disability (1.72 [1.05-2.81], p = 0.031, and 1.97 [1.09-3.59], p = 0.026). In conclusion ADFs of the cervical-upper limb area and trunk area were prognostic factors in ALS patients.**

Keywords: Amyotrophic lateral sclerosis, prognostic factors, needle electromyography, denervation findings, bulbar onset

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a motor neuron disease characterized by serious muscle atrophy and weakness. ALS progresses rapidly with a median survival time from symptom onset of 2-4 years (*1*), and effective treatment has not been established because of the unknown etiology. Adult onset and rapid progression of limb muscle weakness, muscle atrophy, fasciculation, or exaggerated deep tendon reflex lead suspicion of ALS. The diagnosis of ALS is difficult and it is important that the detection of upper or lower motor neuron disorders at each site of the brainstem, cervical, thoracic and lumbosacral spinal cord by taking medical history and physical observation carefully. A variety of prognostic factors for ALS have been reported (*2*), and onset age is a strong prognostic factor in ALS, with decreasing survival time correlating with increasing age of onset (*3-5*). ALS is classified as bulbar onset type, which start with dysarthria, dysphagia, or dyspnea, and extremity onset type, which with muscle weakness or atrophy in an arm or leg. Bulbar onset is associated

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with a worse prognosis than extremity onset (*3,4*).

In Japan, the Specified Disease Treatment Research Program provides a public subsidy for medical expenses for incurable diseases. Patients in each prefecture are required to submit an application form for this program. These forms allow clinical information to be obtained for incurable disease on a national basis, which is useful for studies on epidemiology and pathogenesis, and for evaluation of treatment and drugs (*6*). However, greater emphasis has been placed on administrative applications, while few systematic analyses have been performed for research use. ALS is designated as a specified disease of the program. In this study, we analyzed prognostic factors for progression of ALS using application forms.

2. Methods

2.1. *Patients*

The subjects were patients who registered from 2004 to 2005 in the Japanese Specific Disease Treatment Research Program. Data from application forms for patients who registered with a diagnosis of ALS were submitted to an advisory board on intractable diseases from 47 administrative divisions in Japan. This board included neurologists. These data were evaluated using the diagnostic criteria for ALS defined by the Committee on Intractable Degenerative CNS Diseases of the Ministry of Health and Welfare of Japan (2002), which are based on the diagnostic criteria of the ALS Committee of the World Foundation of Neurology (2000) (*7*) . Patients who received certification then updated their information annually from September to November in future years.

2.2. *Protocol approval and patient consent*

This study conforms to the ethical guidelines for epidemiological research issued by the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare. The ethics committee of the National Institute of Public Health approved this study (NIPH-IBRA No.10021; 10 June, 2010). All data were provided by the Ministry of Health, Labor and Welfare (Notification of Health Service bureau, MHLW; No.0708-1; 8 Jul. 2010). All patients gave consent to utilization of their clinical data in research studies.

2.3. *Definition of variables*

Analysis factors such as sex, onset age, initial symptom at onset, clinical sings at registration, and findings of needle EMG at registration were recorded. The initial symptoms at onset were determined from an interview with the patient or an introductory letter, and included the presence or absence of dysarthria, dysphagia, dyspnea, neck muscle weakness, upper limb muscle weakness, and lower limb muscle weakness. Also, patients were classified into extremity onset type: only muscle weakness at onset, and bulbar onset type that started with dysarthria, dysphagia or dyspnea at onset. Clinical signs at registration were also evaluated by neurologists, and included tongue atrophy, dysarthria, dysphagia, dyspnea, muscle strength, and muscle atrophy. Muscle strength was manually tested and scored using the Medical Research Council (MRC) 6-point scale (range: 0-5) *(8)* in 11 muscle groups: neck flexor muscles; shoulder abductor muscles (right and left); elbow flexor muscles (right and left); wrist flexor muscles (right and left); hip flexor muscles (right and left), and ankle flexor muscles (right and left). A MRC score ≤ 3 was defined as indicating muscle weakness. The presence of muscle atrophy were observed in 10 muscle groups: neck muscle group, upper limb (right and left), girdle muscles (right and left), paraspinal muscles, pelvicrural muscle (right and left), and lower extremities (right and left). Needle electromyography (EMG), a method of puncture of muscles with needle electrodes to record action potentials caused by natural shrinkage and voluntary contraction, was performed in the cranial, cervical-upper limb, trunk, and lumbar-lower limb areas. The presence of active denervation findings (ADFs), defined as fibrillation potentials (Fib-Ps) or positive sharp waves (PSWs); and chronic denervation findings (CDFs), defined as enlarged action potentials and decreased interference patterns, was also evaluated (*9-11*). ADFs are found when innervation of muscles is lost (*12*), and these findings reflect neurodegeneration before appearance of clinical signs such as muscle weakness and muscle atrophy in ALS (*9,13*). CDFs were found when reinnervation occurred following denervation.

Severity of ALS was classified into 5 grades (Table 1) by evaluation of neurological signs at staging and assessment of activities of daily living (ADL) on the modified Rankin Scale (mRS) by the Research Committee of CNS Degenerative Diseases, Ministry of Health, Labour and Welfare. The mRS is a marker of severity of ALS (*14*). In this study, mild ALS was defined as not requiring daily assistance (grades 1 and 2), moderate ALS as requiring daily assistance (grades 3 and 4), and severe ALS as requiring life support such as tubal feeding, gastrostoma, positive pressure ventilation, tracheotomy, and an artificial ventilator (grade 5, Table 1).

2.4. *Outcomes*

Four outcomes were evaluated: time for progression to severe ALS (grade 5, Table 1) as a main outcome and deterioration of ADLs based on loss of speech function, loss of upper limb function, and loss of walking ability

Table 1. Classification of ALS severity by the Research Committee for CNS Degenerative Diseases, Ministry of Health, Labour and Welfare

Severity	Definition
Mild	
Grade 1	Movement disturbance of one extremity or anarthria by bulbar paralysis. No limitation in activities of daily living (ADL).
Grade 2	Apparent movement disturbance in one or two muscle regions in 6 body segments: each limb, trunk, tongue, face, palatal, and pharyngeal region. Slight limitation, but can live an independent life by oneself.
Moderate	
Grade 3	Muscle weakness at more than 3 positions of the above 6 body segments. Cannot do social activities (housework, job) and has mild limitation requiring assistance in ADL.
Grade 4	Inability of any one of breathing, swallowing, or keeping a sitting position. Requires total assistance for ADL.
Severe	
Grade 5	Bedridden, requiring life support including tracheotomy, parenteral nutrition, and an artificial respirator

ALS, amyotrophic lateral sclerosis. The ALS severity classification (grades 1 to 5) is based on evaluation of neurological signs at staging and social life using the modified Rankin scale (mRS). Mild ALS (grades 1 and 2) is defined as not requiring daily assistance. Moderate ALS (grades 3 and 4) is defined as requiring daily assistance. Severe ALS (grade 5) is defined as requiring life support.

as sub outcomes. ADLs were classified into 5 grades referring the Japanese version of the ALSFRS-R (*15*), as validated by Ohashi *et al*. (*16*). The time at which each ADLs deterioration was defined as follows: loss of speech function occurred when the patient lost useful speech; loss of upper limb function occurred when the patient became unable to grip a pen; loss of walking ability occurred when the patient had no purposeful leg movement, respectively.

2.5. *Statistical analysis*

Cox proportional hazards regression analyses were performed for time from registration to progression within 3 years. The hazard ratio (HR) and corresponding 95% confidence interval (CI) and p-value were estimated by Wald test. In univariate analysis, candidate prognostic factors were identified at $p < 0.05$. In multivariate analysis, prognostic factors were selected from these candidate factors using backward selection at $p < 0.05$. To construct a prognostic classification, regression tree analysis for each outcome was performed using prognostic factors as dependent variables. For validation of the prognostic classification (tree structure), regression tree analysis with 1000 bootstrap samples was performed, and the reliability of the crude tree structure was investigated (*17*). The stratified progression-free rates were estimated using the Kaplan-Meier method to show the prognostic classification, and a log-rank test was used for comparison between the stratified groups.

For validation of the severity classification (mild, moderate, and severe), we explored associations with other severity-related measures using a Pearson chi-squared test complemented by Haberman's residual analysis (*18*). To explore the reliability of the severity classification, we analyzed data at the first visit because the number of censors at the last visit was more than that at the first visit. The significance level was $p = 0.05$. All analyses were performed using R ver. 3.1.1. (R Foundation, Austria).

3. Results

3.1. *Patients*

From 2004 to 2005, application forms were submitted by 2,359 patients with ALS, of whom 985 submitted updated application forms for more than one year. All patients fulfilled the diagnostic criteria, as judged by an advisory board. The initial analysis included 959 patients with sporadic ALS, after exclusion of 26 patients with a family history of ALS. Of these 959 patients, 363 had ALS of mild severity and had undergone needle EMG at registration. The characteristics of these patients are shown in Table 2. The patients comprised 218 men and 145 women, and had a median age at disease onset of 62.0 years (range: 18-87 years) and a mean follow-up period of 1.52 ± 0.72 years. The numbers of patients with loss of speech function, loss of upper limb function, and loss of walking ability were 14/363 (3.9%), 6/362 (1.7%), and 0/362 (0%), respectively.

3.2. *Severity classification*

The classification of mild, moderate and severe ALS was validated based on significant associations found with measures related to progression of ALS, including levels of speech function ($p < 0.001$), upper limb function ($p < 0.001$), and walking ability ($p < 0.001$), and the number of areas with muscle weakness ($p < 0.001$) and muscle atrophy ($p < 0.001$) (Table 3).

3.3. *Time to progression*

Of the patients with initially mild ALS, 38.3% (139/363) had progressed severe ALS at the last follow-up. The rate of patients with loss of speech function, loss of upper limb function, and loss of walking ability at the last follow-up were 31.1% (113/363), 30.6% (111/363), and 22.0% (80/363), respectively.

The results of univariate regression analysis of the

Table 2. Baseline characteristic of ALS patients

Variable (n = 363)	Number of patients	%
Gender: male	145/363	39.9
Age at onset (years old)		
≤ 40	11/363	3.0
41-64	220/363	60.6
≥ 65	132/363	36.4
Onset type : Bulbar onset	222/360	61.7
Tongue atrophy at registration: presence	172/361	47.6
Dysarthria at registration: presence	193/363	53.2
Dysphagia at registration: presence	145/361	40.2
Dyspnea at registration: presence	50/357	14.0
Neck flexors strength at registration: with muscle weakness	47/358	13.1
Shoulder abductors strength at registration: with muscle weakness	107/360	29.7
Elbow flexors strength at registration: with muscle weakness	94/359	26.2
Wrist extensors strength at registration: with muscle weakness	77/359	21.4
Hip flexors strength at registration: with muscle weakness	39/359	10.9
Ankle extensors at registration: with muscle weakness	63/352	17.9
Active denervation findings at registration: presence		
Cranial area	96/363	26.4
Cervical-upper limb area	226/363	62.3
Trunk area	42/363	11.6
Lumbar-lower limb area	161/363	44.4
Chronic denervation findings at registration: presence		
Cranial area	154/363	42.4
Cervical-upper limb area	294/363	81.0
Trunk area	73/363	20.1
Lumbar-lower limb area	254/363	70.0

ALS: amyotrophic lateral sclerosis.

Table 3. Associations between severity classification and other severity-related measures

Variable	Category			p value
	Mild	Moderate	Severe	
Speech function				
1	59*	53*	11	< 0.001
2	43*	33	17	
3	13	9	9	
4	7	12	10	
5	11	16	58*	
Upper limb function (handwriting)				< 0.001
1	59*	11	9	
2	56*	26	28	
3	8	23*	12	
4	8	29*	16	
5	2	34*	41*	
Walking ability				< 0.001
1	54*	15	11	
2	50*	22	25	
3	28	39*	17	
4	1	37*	18	
5	0	11	34*	
Number of areas with muscle weakness				< 0.001
≤ 1	71*	13	15	
2-5	41*	29	17	
6-8	12	25	23	
9-11	5	43*	43*	< 0.001
Number of areas with muscle atrophy				
≤ 1	28*	1	7	
2-4	50*	23	14	
5-7	31	39*	20	
8-10	24	61*	65*	

ALS: amyotrophic lateral sclerosis. The significance of the association between severity and each index of ALS progression was assessed using a Pearson chi-squared test of independence. Residual analysis was also performed for identifying the categories responsible for a significant chi-square statistic. * indicates a significant large number ($p < 0.05$).

time to progression to severe ALS are shown in Table 4. In this analysis, the candidate prognostic factors were bulbar onset (HR: 2.28 [95% CI: 1.63-3.19], $p < 0.001$), tongue atrophy at registration (2.26 [1.60-3.19], $p < 0.001$), dysarthria at registration (2.23 [1.56-3.18], $p < 0.001$), dysphagia at registration (2.25 [1.61-3.15], $p < 0.001$), dyspnea at registration (2.00 [1.33-3.00], $p = 0.001$), ADFs of the cervical-upper limb area at registration (1.59 [1.10-2.29], $p = 0.013$), and CDFs of the cervical-upper limb area at registration (1.41 [1.01–1.96], $p = 0.044$). The results of univariate regression analysis of the times to loss of speech function, loss of upper limb function and loss of walking ability are also shown in Table 4.

The results of multivariate regression analysis of the time to progression to severe ALS are shown in Table 5. Bulbar onset (1.68 [1.13-2.49], $p = 0.010$), tongue atrophy at registration (1.69 [1.14-2.51], $p = 0.009$), dyspnea at registration (1.57 [1.02-2.41], $p = 0.042$), and ADFs of the cervical-upper limb area at registration (1.81 [1.25-2.63], $p = 0.002$) emerged as prognostic factors for time for progression to severe ALS. The results of regression tree analysis are shown in Figure 1A as the stratified progression-free rate. ADFs of the cervical-upper limb area were found to be significant in progression to severe ALS. The results of multivariate analysis and regression tree analysis for the times to the three sub outcomes are shown in Table 5 and Figure 1B-D. These results indicated that ADFs of the trunk area were prognostic factors for upper limb dysfunction and walking disability.

Table 4. Univariate Cox regression analyses for times to loss of speech function, loss of walking ability, and loss of upper limb function

Variable	Progression to severe			Loss of speech function			Loss of upper limb function			Loss of walking ability		
	HR	95% CI	*p* value	HR	95% CI	*p* value	HR	95% CI	*p* value	HR	95% CI	*p* value
Gender (men)	1.01	0.72 - 1.43	0.936	0.66	0.46 - 0.96	0.031	1.36	0.91 - 2.02	0.131	1.40	0.86 - 2.25	0.175
Age at onset	1.01	1.00 - 1.03	0.167	1.03	1.01 -1.05	0.003	0.99	0.97 - 1.00	0.103	0.99	0.97 - 1.01	0.428
Bulbar onset	2.28	1.63 - 3.19	< 0.001	5.69	3.78 – 8.54	< 0.001	0.55	0.36 - 0.84	0.005	1.17	0.75 - 1.84	0.492
Tongue atrophy at registration	2.26	1.60 - 3.19	< 0.001	2.58	1.74 - 3.83	< 0.001	0.72	0.49 - 1.06	0.092	0.94	0.61 - 1.47	0.793
Dysarthria at registration	2.23	1.56 - 3.18	< 0.001	5.10	3.17 - 8.19	< 0.001	0.69	0.47 - 1.00	0.048	0.94	0.61 - 1.46	0.781
Dysphagia at registration	2.25	1.61 - 3.15	< 0.001	4.49	3.01 - 6.68	< 0.001	0.63	0.42 - 0.95	0.026	1.06	0.68 - 1.67	0.795
Dyspnea at registration	2.00	1.33 - 3.00	0.001	1.92	1.21 - 3.04	0.006	0.88	0.51 - 1.52	0.637	1.39	0.79 - 2.43	0.258
Neck flexors strength at registration	1.36	0.86 - 2.14	0.187	0.94	0.54 - 1.64	0.825	1.15	0.68 - 1.96	0.604	1.20	0.65 - 2.21	0.570
Shoulder abductors strength at registration	1.06	0.74 - 1.51	0.760	0.56	0.36 - 0.87	0.010	2.54	1.75 - 3.68	< 0.001	1.05	0.66 - 1.68	0.824
Elbow flexors strength at registration	0.87	0.59 - 1.28	0.475	0.47	0.29 - 0.77	0.003	2.20	1.50 - 3.21	< 0.001	0.85	0.52 - 1.41	0.538
Wrist extensors strength at registration	0.99	0.66 - 1.51	0.973	0.59	0.35 - 1.00	0.050	3.01	2.03 - 4.48	< 0.001	1.23	0.72 - 2.08	0.451
Hip flexors strength at registration	0.80	0.44 - 1.45	0.462	0.54	0.25 - 1.16	0.113	0.85	0.44 - 1.62	0.614	2.00	1.10 - 3.63	0.023
Ankle extensors at registration	0.58	0.34 - 0.99	0.047	0.60	0.33 - 1.09	0.10	0.66	0.37 - 1.18	0.163	1.58	0.92 - 2.70	0.097
Active denervation findings at registration												
Cranial area	1.45	1.02 - 2.06	0.039	2.07	1.43 - 3.02	< 0.001	1.02	0.67 - 1.55	0.925	1.00	0.61 - 1.62	0.990
Cervical-upper limb area	1.59	1.10 - 2.29	0.013	0.95	0.65 - 1.38	0.777	1.90	1.24 - 2.92	0.003	1.07	0.68 - 1.70	0.772
Trunk area	1.12	0.68 - 1.87	0.652	0.80	0.43 - 1.49	0.479	1.96	1.20 - 3.18	0.007	1.94	1.09 - 3.45	0.025
Lumbar-lower limb area	0.96	0.69 - 1.34	0.809	0.69	0.47 - 1.01	0.061	1.30	0.90 - 1.90	0.162	1.47	0.95 - 2.28	0.086
Chronic denervation findings at registration												
Cranial area	1.41	1.01 - 1.96	0.044	1.99	1.37 - 2.89	< 0.001	0.76	0.51 - 1.12	0.162	0.96	0.61 - 1.50	0.848
Cervical-upper limb area	1.59	0.98 - 2.58	0.060	1.05	0.65 - 1.70	0.852	1.45	0.85 - 2.46	0.169	1.24	0.68 - 2.24	0.484
Trunk area	1.14	0.76 - 1.71	0.537	0.88	0.54 - 1.43	0.603	1.00	0.63 - 1.60	0.987	1.06	0.61 - 1.84	0.826
Lumbar-lower limb area	1.01	0.70 - 1.44	0.969	0.64	0.44 - 0.93	0.019	0.93	0.62 - 1.39	0.722	1.31	0.80 - 2.15	0.278

HR: Hazard Ratio; CI: Confidence Interval; MRC: Medical Research Council; EMG: electromyography. The p-value and 95%CI were calculated using a Wald test. Variable (reference); Gender (women), Age at onset (1), onset type (extremity onset)), tongue atrophy at registration (absence), dysarthria at registration (absence), dysphagia at registration (absence), dyspnea at registration (absence), muscle strength at registration (no muscle weakness: MRC score ≤ 3), EMG finding at registration (absence). In an area tested on the right and left, the lower muscle strength was used for analysis.

Table 5. Multivariate Cox regression analyses for time to progression to severe ALS, tongue atrophy and dysarthria

Variable	Progression to severe			Loss of speech function			Loss of upper limb function			Loss of walking ability		
	HR	95% CI	*p* value	HR	95% CI	*p* value	HR	95% CI	*p* value	HR	95% CI	*p* value
Gender (men)												
Age at onset												
Bulbar onset	1.68	1.13 - 2.49	0.010*	3.81	2.24 - 6.48	< 0.001*						
Tongue atrophy at registration	1.69	1.14 - 2.51	0.009									
Dysarthria at registration												
Dysphagia at registration				1.87	1.11 - 3.14	0.018*						
Dyspnea at registration	1.57	1.02 - 2.41	0.042*									
Neck flexors strength at registration												
Shoulder abductors strength at registration							1.91	1.25 - 2.91	0.003*			
Elbow flexors strength at registration												
Wrist extensors strength at registration							2.12	1.35 - 3.32	0.001*			
Hip flexors strength at registration										1.88	1.05 - 3.55	0.033*
Ankle extensors at registration												
Active denervation findings at registration												
Cranial area	1.81	1.25 - 2.63	0.002*									
Cervical-upper limb area							1.72	1.05 - 2.81	0.031*	1.97	1.09 - 3.59	0.026
Trunk area												
Lumbar-lower limb area												
Chronic denervation findings at registration												
Cranial area												
Cervical-upper limb area												
Trunk area												
Lumbar-lower limb area												

HR: Hazard Ratio; CI: Confidence Interval; MRC: Medical Research Council; EMG: electromyography. The p-value and 95%CI were calculated using a Wald test. Variable (reference); Gender (women), Age at onset (1), Onset type (extremity onset), tongue atrophy at registration (absence), dysarthria at registration (absence), dysphagia at registration (absence), dyspnea at registration (absence), muscle strength at registration (no muscle weakness: MRC score ≤ 3), EMG finding at registration (absence). In an area tested on the right and left, the lower muscle strength was used for analysis. Variables marked with * were chosen as results of regression tree analysis.

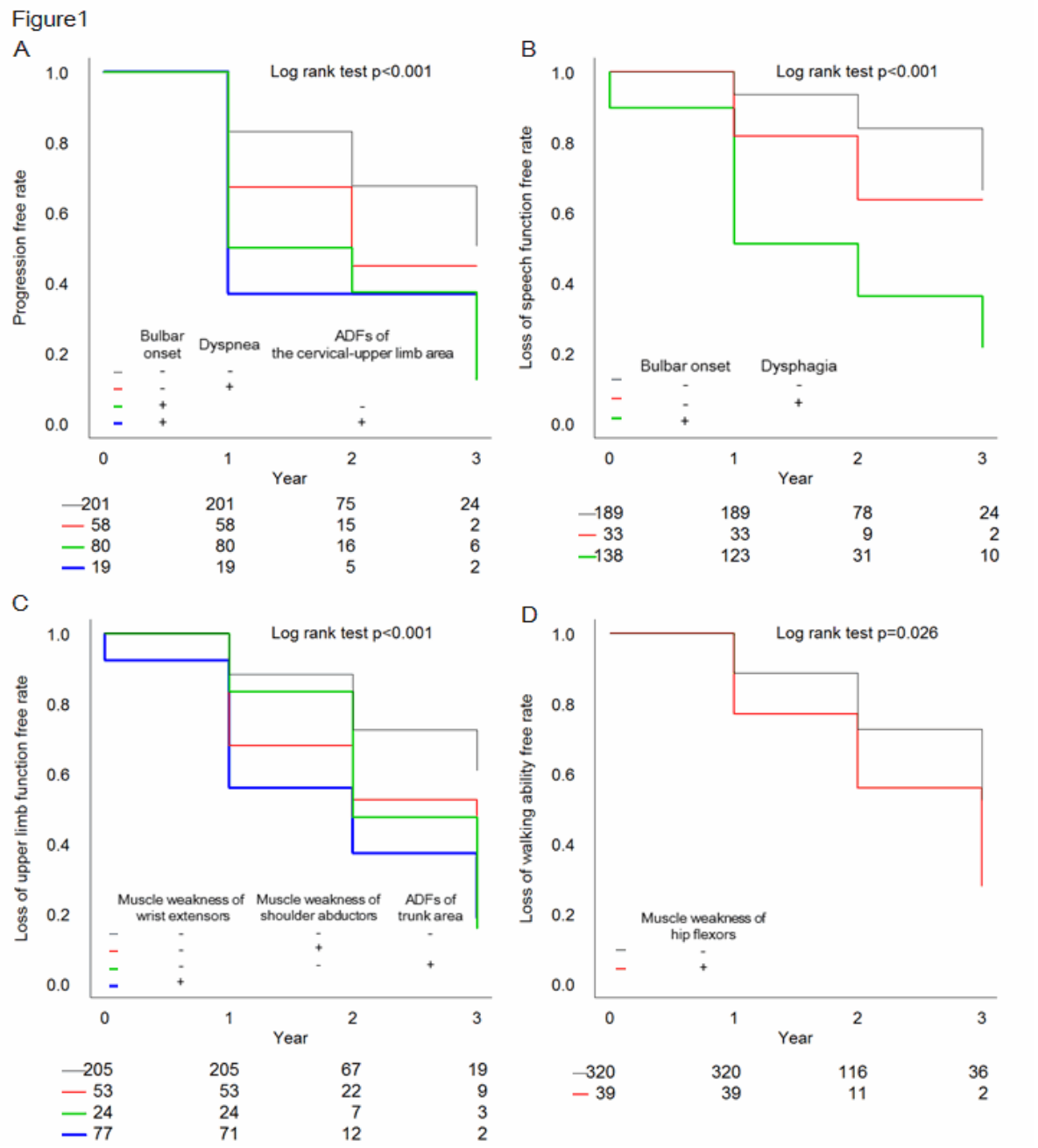

Figure 1. Stratified Kaplan-Meier curves for four outcomes based on regression tree analysis. Curves are shown for progression to severe ALS **(A)**, loss of speech **(B)**, loss of upper limb function **(C)**, and loss of walking ability **(D)**. The curves were compared by log-rank test. For progression to severe ALS, patients were classified into 5 groups based on onset type (+: bulbar onset, -: extremity onset), presence or absence of respiratory problems at registration, and ADFs of the cervical-upper limb area at registration **(A)**. For loss of speech, patients were classified into 3 groups based on onset type and presence or absence of dysphagia at registration **(B)**. For loss of upper limb function, patients were classified into 4 groups based on presence or absence of weakness of wrist extensors at registration, of shoulder abductors at registration, and ADFs of the trunk area at registration **(C)**. For loss of walking ability, patients were classified into 2 stratified groups based on presence or absence of weakness of hip flexors at registration **(D)**. The stratified Kaplan-Meier curve for each outcome was well-distinguished by log-rank test (**A**: $p < 0.001$, **B**: $p < 0.001$, **C**: $p < 0.001$, **D**: $p = 0.026$).

4. Discussion

In this study we tried to examine prognostic factors for progression ALS by interannual analysis of application forms, which provided important medical data on a national scale, focusing on patients with initial mild ALS who did not require daily assistance. The main outcome in our study: progression to severe ALS, is a stage at which a patient requires a ventilator and parenteral nutrition for life support. Thus an observation period of 3 years should be sufficient to analyze prognostic factors for ALS considering median survival time of 2-4 years (*1*).

Our results indicated that bulbar onset, tongue atrophy, dyspnea, and ADFs of the cervical-upper limb area were prognostic factors for progression from mild

to severe ALS (Table 5). Many studies reported bulbar onset and tongue atrophy were important prognostic factors (*19*), however a few studies reported the possibility of ADFs as prognostic factors of ALS (*20*).

Progression to severe ALS is associated with decreased swallowing function and respiratory function. Swallowing is controlled by muscles that are innervated mainly by the pons and medulla oblongata such as the glossopharyngeal nerve, vagal nerve and hypoglossal nerve (*21,22*), and bulbar onset and tongue atrophy are associated with loss of this function. Breathing is controlled by complex relationships among many muscles, of which the diaphragm and the anterior, middle and posterior scalene muscles, which function in intake, are innervated by C3-C4, C4-C7, C2-C7 and C5-C8, respectively. Muscles innervated by the brachial plexus (C5-T1) are tested in needle EMG of the cervical-upper limb area. Then, ADFs in this area might reflect neurodegeneration of muscles involved in respiratory functions. ADFs are said to show neurodegeneration from before appearance of clinical signs such as muscle weakness and muscle atrophy in ALS (*10,14*). Therefore we analyzed the prognosis of patients without neck flexors muscle weakness nor shoulder abductors muscle weakness at registration and found that the number of patients with ADFs of the cervical-upper limb area who progressed to severe ALS within 3 years were significant large ($\chi^2 = 4.00$, p = 0.045).

Figure 1A suggested that patients with both bulbar onset and ADFs of the cervical-upper limb area had poor prognosis. Further analysis found that of patients with extremity onset, the number of patients with ADFs of the cervical-upper limb area who progressed to severe ALS within 3 years were significant large ($\chi^2 = 3.89$, p = 0.049). This suggested that ADFs of the cervical-upper limb area were also important for predicting prognosis ALS in patients with extremity onset.

Furthermore, ADFs in the trunk area were prognostic factors for upper limb dysfunction and walking disability (Table 5). In needle EMG of the trunk area, muscles of the thoracic spinal cord are tested, including the paraspinal muscles and abdominal rectus muscle (*23,24*). Degeneration of motor neurons may spread contiguously throughout the three-dimensional anatomy of connected and neighboring neurons in ALS (*25,26*), and this may explain upper limb dysfunction resulting from proximal progression of denervation of the trunk area and walking disability due to distal progression.

In this study, CDFs were not prominent as risk factors. CDFs were found when reinnervation occurred following denervation, but occasionally did not occur, especially in extremely in cases with fast progression. Furthermore, the Awaji criteria (2008) indicate that detection of fasciculation potential (FP), which was not investigated in this study, in muscle with chronic findings carries the same significance as active findings such as Fib-Ps and PSWs (*10,27*). Previous study said that FP is a specific finding in ALS, and occurs inconsistently in the initial stage of ALS before appearance of Fib-Ps and PSWs (*28*). We performed multivariate regression analysis and identified the detection of either ADFs or CDFs of the cervical-upper area as prognostic factors for progression to severe ALS (3.00 [1.51-5.94], $p = 0.002$). Interpretation of these associations with CDFs requires further studies.

There are other limitations in this study, including the short follow-up period and ambiguity of the time to outcomes. However, of the patients with initially mild ALS, 38.3% had severe ALS at the last follow-up. Thus, due to the large number of events, our prognostic analysis has certain reliability.

ALS causes a lethal respiratory failure but, recently many patients introduce an artificial respiratory management. However, much previous reports on prognostic factors were set an outcome as time to death, which could not predict the degree of ALS progress. In this study we identified needle EMG findings as prognostic factors, which closely associated with the pathology of ALS, based on nationwide data of ALS patients. The needle EMG is an invasive diagnostic procedure, however, our findings suggest that this experiment is useful for not only accurate diagnosis ALS (*29,30*) but also prediction ALS prognosis or progression.

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Original Article

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Multiplex cytokine analysis of Werner syndrome

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Summary **We reported a minor inflammation-driven ageing (inflammageing) assessed by highly sensitive CRP (hsCRP) in normal individuals and patients with Werner syndrome (WS), followed by an ageing associated Th2-biased cytokine change in normal ageing in the previous papers. To further study the association of hsCRP and 26 cytokines/chemokines in 35 WS patients, a multiple cytokine array system was used in the same serum samples as were examined for hsCRP. The serum levels of Th2 cytokines (IL-4, IL-6, IL-10, and GM-CSF), Th1 products (IL-2, TNFα, IL-12, and IFNγ) and monocyte/macrophage products (MCP-1, basic FGF and G-CSF) in WS were significantly elevated compared with normal ageing. Elevated hsCRP level in WS was significantly correlated with IL-6, IL-12 and VEGF levels, if age and sex were taken into account. A pro-inflammatory cytokine/chemokine circuit-stimulated immunological shift to Th2 in WS was similar to normal ageing. These cytokine/chemokine changes may induce a systemic chronic inflammation monitored by hsCRP, though these immunological changes in WS were more complicated than normal ageing, possibly due to the WS-specific chronic inflammation such as skin ulcer, diabetes mellitus and central obesity with visceral fat deposition. Further study may warrant the pathophysiology of Th2 shift and Th2-biased inflammageing in normal ageing and WS.**

Keywords: Ageing, inflammageing, Werner syndrome, CRP, cytokine, chemokine

1. Introduction

Werner syndrome (WS; MIM#27770), the genetically-determined progeroid syndrome has been extensively studied as the representative natural model of human ageing. We have reported the elevation of inflammatory markers in WS in a series of publications irrespective of the apparent inflammation (*1*).

Our recent study indicated a significant ageing-associated increase in the serum level of highly sensitive CRP (hsCRP) in the normal Japanese individuals and the serum hsCRP level was also significantly elevated in WS compared with age-matched normal adult (NA) control and normal elderly (NE) population from both sexes (*2*).

Human ageing is inevitably accompanied by an increasing chance of environmental attack from inside (such as mutants, endoplasmic reticulum (ER) stress and by-products associated with immune- surveillance activity) and outside (such as ultra violet light, air pollution, allergens, infectious agents, drugs and foods), producing a minor inflammation that is widely recognized as a patho-physiologically fundamental metabolism to generate energy with thermogenesis, leading to tissue development, wound healing or tissue destruction during healthy development and ageing (*3-6*).

Ageing-associated inflammation coined as "inflammageing" has been monitored mainly by hsCRP (*7-9*). Inflammageing is probably caused by an imbalance

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between an increase in pro- and a decrease in anti-inflammatory cytokines/chemokines, leading to ignite the ageing-related conditions including diabetes mellitus (DM), sarcopenia, osteoporosis, cancer, atherosclerosis, cognitive decline and finally death (*3,8,10*).

Ageing-associated changes of pro/anti-inflammatory cytokines/chemokines have been reported by using ELISA and multiplex technology. However, the results are conflicting. Ageing-associated elevation of pro-inflammatory cytokines/chemokines including interleukin (IL)-6, IL-8 (CXCL8), tumor necrosis factor α (TNFα), macrophage inhibitory protein-1α (MIP-1α: CCL3) and monocyte chemoattractant protein-1 (MCP-1: CCL2) was reported by Mariani *et al* (*11*). However, both Shurin *et al*. (*12*) and Kim *et al*. (*13*) described no ageing-associated changes of these cytokines/chemokines. Shurin *et al*. (*12*) reported a significant age-associated increase of interferonγ inducible protein-10 (IP-10: CXCL10) and eotaxin (CCL11). Elevation of IL-6, MCP-1 and IP-10 was described by Miles *et al*. (14), Inadera *et al*. (*15*) and Antonelli *et al*. (*16*).

In our preceding paper, the serum levels of IL-4, IL-6, IL-13, IL-15, granulocyte-macrophage- colony stimulating factor (GM-CSF), interferon-γ (IFNγ), IP-10 and TNFα were significantly correlated with normal ageing (manuscript submitted). In contrast, IL-2, IL-8, MIP-1α levels were negatively associated with normal ageing. The Th2 products: IL-6 and IL-13 levels were significantly associated with serum level of hsCRP in normal ageing, if age and sex were taken into account. Cytokine/chemokine analysis in WS has never been reported.

The aim of this study was to *i*) compare the serum levels of 26 cytokines/chemokines examined by multiplex assay in WS with the apparently healthy Japanese volunteers; and *ii*) clarify the association of 26 cytokines/chemokines with the increased level of hsCRP in WS.

2. Materials and Methods

2.1. *Study population*

All the samples studied in the present experiment were collected between 2000 and 2010, and were the same sera as were used in the previous hsCRP study (*2*). A total of 35 serum samples from the patients with mutation-proven WS aged between 32 and 70 years were used. All of the WS patients showed the characteristic manifestations as previously described: typical body status/face, hoarseness, gray hair/alopecia, skin hyper/hypo-pigmentation, sarcopenia, cataract, osteoporosis, and subcutaneous calcification. As some WS patients had diabetes mellitus (DM) and skin ulcers (SU), the patients were sub-grouped into 1) SU (+) DM (+) (*n* = 14), 2) SU (+) DM (-) (*n* = 12), 3) SU (-) DM (+) (*n* = 4), and 4) SU (-) DM(-) (*n* = 5) based on their phenotypes as indicated in Table 1.

Table 1. Clinical characteristics in Werner syndrome patients

Subgroups	SU*	DM**	ID	Age	Sex
1	+	+	WS12901	32	F
1	+	+	WS57201	37	M
1	+	+	WS19201	38	M
1	+	+	WS56301	39	M
1	+	+	WS57801	41	M
1	+	+	WS51301	42	M
1	+	+	WS19201	44	M
1	+	+	WS6301	46	M
1	+	+	WS53601	46	M
1	+	+	WS58501	51	M
1	+	+	WS58301	53	M
1	+	+	WS54801	57	M
1	+	+	WS56201	70	M
1	+	+	WS1801	70	M
2	+	-	WS6103	32	M
2	+	-	WS6104	32	M
2	+	-	WS14501	35	M
2	+	-	WS51601	36	F
2	+	-	WS53101	38	F
2	+	-	WS53901	43	F
2	+	-	WS53801	46	F
2	+	-	WS2101	50	F
2	+	-	WS55801	53	F
2	+	-	WS52901	54	F
2	+	-	WS54001	57	F
2	+	-	WS4701	59	F
3	-	+	WS58701	35	M
3	-	+	WS57701	38	F
3	-	+	WS57401	41	M
3	-	+	WS4401	41	M
4	-	-	WS5801	43	M
4	-	-	WS0402	47	M
4	-	-	WS7501	48	M
4	-	-	WS0401	49	F
4	-	-	WS10501	52	F

*SU: skin ulcer. **DM: diabetes mellitus. 26 had skin ulcers (SU) and 18 diabetes mellitus (DM). Subgroups: 1) SU (+) DM (+), *n* = 14; 2) SU (+) DM (-), *n* = 12; 3) SU (-) DM (+), *n* = 4; 4) SU (-) DM (-), *n* = 5.

All of the individuals provided written informed consent for this study, which was approved by the ethics committee of Toin University of Yokohama. All of the samples were stored at -80°C until use. For statistical comparison, 113 normal individuals were divided into two groups according to their age: normal adult (NA) aged between 25 and 70 years (*n* = 57; M = 29, F = 28) and normal elderly (NE) aged between 71 and 100 years (*n* = 56; M = 12, F = 44). All the normal individuals including NE were the same as examined in the previous study and met the SENIEUR criteria (*17*).

2.2.1. *Multiplex cytokine array system*

Serum levels of 26 cytokines/chemokines including IL-1β, IL-1 receptor antagonist (ILra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, basic fibroblast growth factor (basic FGF), granulocyte-colony stimulating factor (G-CSF), GM-CSF, platelet derived growth factor (PDGF), vascular endothelial

growth factor (VEGF), TNFα, IFNγ, IL-8, IP-10, MCP -1, MIP-1α, MIP-1β (CCL4) and eotaxin were simultaneously measured using commercially-available bead-based immunofluorescence Bio-Plex Suspension Array System (BioRad; Hercules, CA) according to the manufacturer's instruction.

Briefly, six distinct sets of fluorescently dyed beads loaded with capture monoclonal antibodies specific for each cytokine/chemokine to be tested, were used. Serum samples (50 µL/well of fourfold diluted serum) or standards (50 µL/well) were incubated with 50 µL of premixed bead sets into the pre-wetted 96 well microtiter plates at 4°C. After incubation and washing, 25 µL of fluorescent detection antibody mixture was added for 30 min and then the samples were washed and resupended in assay buffer.

High standard curves for each soluble factor were used. The low standard curves were obtained by tenfold diluted high standards. The formation of different sandwich immune-complexes was obtained by using the Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad; Hercules, CA). A 50 µL volume was sampled by each well and the fluorescent signal of a minimum of 100 beads per region (cytokine/chemokine) was evaluated and recorded. Values presenting a coefficient of variation beyond 10% were discarded before the final analysis.

2.2.2. *Determination of hsCRP*

The data of hsCRP used in this study was obtained in the previous experiment (*2*) by using CircuLex high-sensitivity CRP ELISA kit (MBL Woburn, MA) according to the user's manual.

2.3. *Data analysis and statistics*

Differences of serum cytokines/chemokines between WS and healthy individual groups (NA and NE) were evaluated by Wilcoxon rank sum test. We examined ageing-associated changes of serum levels of cytokines/chemokines using regression analyses expressed as

$$\log\_e(\text{cytokine/chemokine}(j)) = a + b^{*}\,\text{Age},$$

where a is an estimated intercept, b is an estimated regression coefficient for Age and j is an indicator for individual cytokine/chemokine. To examine the relationship between serum levels of hsCRP and cytokine/chemokine, we performed regression analyses expressed as

$$\log\_e(\text{hsCRP}) = a + b^{*}\log\_e(\text{cytokine/chemokine}(j)),$$

where a is an estimated intercept, b is an estimated regression coefficient and j is an indicator for individual cytokine/chemokine. Multiple regression models were used to further examine the relationship between hsCRP and cytokines/chemokines with adjustment of sex and age effects on the serum levels. The model (a) was expressed as

$$\log\_e(\text{hsCRP}) = a + b_1{*}\text{Age} + b_2{*}\text{Sex} + b_3{*}\log\_e(\text{cytokine/chemokine}(j)),$$

where a (intercept), b_1, b_2 and b_3 are estimated regression coefficients and j is an indicator for individual cytokine/chemokine. The model (b) was expressed as

$$\text{hsCRP} = \exp\{a + b1{*}\text{Age} + b2{*}\text{Sex} + b3\text{cytokine/chemokine}(j)\},$$

where a (intercept), $b1$,$b2$ and $b3$ are estimated regression coefficients and j is an indicator for individual cytokine/chemokine. We used Akaike's Information Criterion (AIC) (*18*) for model selection between models with original data and models with log-transformed values (not shown). We show only results based on models with log-transformed values described above because they were better than models with original data. Statistical language R (*19*) was used for the analyses. p-values < 0.05 were considered to be statistically significant. Differences of serum cytokine/chemokine levels between subgroups in WS were estimated by Welch's two-sample t-test with unequal variances.

Serum cytokine/chemokine data were analyzed using the Bio-Plex manager software version 5.0 (Bio-Rad; Hercules, CA). Standard levels between 70 and 130% of the expected values were considered to be accurate and were used. In general, at least six standards were accepted and used to establish standard curves following a five-parameter logistic regression model (5PL), Sample concentrations were immediately interpolated from the standard curves. Values were expressed as pg/mL and presented as mean ± S.E.

3. Results

3.1. *Cytokine/chemokine levels between Normal adult (NA) and Normal elderly (NE) groups*

As indicated in Table 2, serum levels of IL-4, IL-6, IL-13, IL-15, GM-CSF, IP-10, MCP-1 and TNFαin NE group were significantly elevated compared with NA group. In contrast, serum levels of IL-1β, IL-1ra and MIP-1αin NE group were significantly decreased compared with NA group. The rest of the cytokine/chemokine levels examined were comparable between NA and NE.

3.2. *Cytokine/chemokine levels in WS*

Single regression analyses of 26 cytokines/chemokines

Table 2. Cytokine/chemokine changes between NA, NE and WS

Cytokines/chemokines (pg/mL)	Normal adult (NA) (mean ± S.E.; *n* = 35)	WS (mean ± S.E.; *n* = 35)	Normal elderly(NE) (mean ± S.E.; *n* = 56)	*p* value		
				NA *vs.* NE	WS *vs.* NA	WS *vs.* NE
IL-4	6.4 ± 0.4	8.8 ± 0.9	8.6 ± 0.9	0.008**	0.008**	0.113
IL-6	11.6 ± 3.2	408.6 ± 381.5	22.1 ± 8.1	< 0.001***	< 0.001***	0.007**
IL-13	9.7 ± 1.0	13.9 ± 1.8	16.7 ± 2.0	0.002**	0.057	0.429
IL-15	4.4 ± 0.8	7.5 ± 1.2	9.5 ± 0.9	< 0.001***	< 0.001***	0.046*
GM-CSF	33.9 ± 9.9	117.4 ± 37.5	99.6 ± 17.4	< 0.001***	< 0.001***	0.932
IP-10 (CXCL10)	862.4 ± 51.2	1898.3 ± 530.8	1503.6 ± 175.6	< 0.001***	0.164	0.083
MCP-1 (CCL2)	46.1 ± 6.2	53.2 ± 4.1	51.5 ± 4.5	0.010**	0.008**	0.431
TNFα	32.9 ± 9.1	39.9 ± 7.8	38.1 ± 7.6	0.020*	0.002**	0.113
IL-5	2.53 ± 0.62	2.67 ± 0.31	2.82 ± 1.05	0.245	0.078	0.007**
IL-9	38.37 ± 17.08	101.07 ± 71.8	42.04 ± 9.78	0.328	0.121	0.549
IL-10	2.59 ± 0.34	3.66 ± 0.45	2.93 ± 0.31	0.303	0.031*	0.149
basicFGF	22.09 ± 1.54	31.48 ± 2.72	24.47 ± 2.45	0.982	0.002**	0.004**
G-CSF	10.80 ± 0.69	16.84 ± 1.58	14.73 ± 4.31	0.673	< 0.001***	< 0.001***
IFNγ	158.97 ± 31.48	192.9 ± 22.58	226.36 ± 45.16	0.086	0.021*	0.259
VEGF	114.68 ± 9.99	247.4 ± 51.29	165.56 ± 15.31	0.074	0.094	1
Eotaxin (CCL11)	106.31 ± 7.48	94.45 ± 12.0	117.63 ± 9.23	0.282	0.278	0.079
IL-1β	6.4 ± 4.0	10.8 ± 8.7	1.7 ± 0.4	0.041*	0.502	0.005**
IL-1ra	59.9 ± 4.6	72.4 ± 8.9	48.6 ± 5.4	0.024*	0.355	0.011*
IL-2	3.8 ± 0.7	5.07 ± 1.37	2.99 ± 0.97	0.373	0.029*	< 0.001***
IL-7	8.1 ± 0.71	10.93 ± 1.37	7.92 ± 0.44	0.469	0.128	0.157
IL-12	19.82 ± 1.45	30.42 ± 5.09	18.35 ± 1.72	0.26	0.303	0.091
IL-17	28.56 ± 2.08	31.23 ± 3.23	25.58 ± 1.49	0.25	0.213	0.071
MIP-1α (CCL3)	9.6 ± 1.9	5.6 ± 0.6	4.9 ± 0.7	< 0.001***	0.247	0.053
MIP-1β (CCL4)	209.27 ± 47.63	125.37 ± 10.93	133.77 ± 7.29	0.308	0.772	0.455
IL-8 (CXCL8)	164.51 ± 83.30	17.63 ± 1.88	34.76 ± 15.81	0.172	0.262	0.189
PDGF	18015.1 ± 2613.1	7482.1 ± 887.2	12540.5 ± 1475.7	0.877	0.082	0.007**

Cytokine/chemokine levels between NA, NE and WS were tested by Wilcoxon rank sum test: *:$p < 0.05$, **:$p < 0.01$, ***:$p < 0.001$.

showed no significant age-associated changes in WS, as was already inferred in the previous hsCRP paper (*2*).

In WS patients, serum levels of IL-2, IL-6, basic FGF, and G-CSF were significantly elevated compared with NA and NE (Table 2). Levels of IL-4, IL-15, GM-CSF, MCP-1, TNFα, IL-10 and IFNγin WS were significantly increased compared with NA. Both IL-1β and IL-1ra levels were significantly elevated in WS in comparison with NE. Serum levels of PDGF, IL-5 and IL-15 in WS were significantly decreased compared with NE. The rest of the cytokine/chemokine levels (IL-7, IL-8, IL-9, IL-12, IL-17, eotaxin and MIP-1β) were comparable between NA, NE and WS.

3.3. *Association of cytokine/chemokine with serum hsCRP in WS*

Using multiple regression models, temporal effect of age on the serum level of hsCRP was determined. The Table 3-a and 3-b showed estimated regression coefficients with S.E., and *p*-values.

IL-6 was significantly associated with hsCRP. The relationship between IL-6, hsCRP and ageing according to the model (a) was log_e (hsCRP) = -1.199 + 0.04 × Age + 0.339 × Sex + 0.317 × log_e (IL-6) (Table 3-a).

Both IL-12 and VEGF were also significantly associated with hsCRP according to the model (b); hsCRP = exp{1.311 + 0.01 × Age + 0.522 × Sex + 0.007 × IL-12} and hsCRP = exp{1.26+ 0.01 × Age + 0.64 × Sex + 0.001 × VEGF} (Table 3-b), respectively. In these formulae, Sex was 1 for male and 0 in female. No sex difference was observed concerning to the ageing associated changes of 26 cytokines/chemokines examined.

3.4. *Association of cytokines/chemokines with clinical phenotypes in WS*

In the WS patients, the serum hsCRP level was similar between SU (+) and SU (-) groups or DM (+) and DM (-) groups, and among SU (+) DM (+), SU (+) DM (-) , SU (-) DM (+) and SU (-) DM(-) subgroups, as was reported in the previous paper (*2*).

Significant differences were in eotaxin (111.8 ± 14.0 *vs*. 44.4 ± 13.2 pg/mL, $p < 0.05$), IP-10 (2348.6 ± 693.2 *vs*. 597.4 ± 183.2 pg/mL, $p < 0.05$) and MIP-1α (6.2 ± 0.8 *vs*. 3.9 ± 0.7 ng/mL, $p < 0.05$) between SU (+) and SU (-), respectively. Serum levels of eotaxin (121.0 ± 17.6 *vs*. 66.3 ± 13.4 pg/mL, $p < 0.05$) and G-CSF (20.4 ± 2.1 *vs*. 13.1 ± 2.1 pg/mL, $p < 0.01$) were significantly elevated in DM (+) group compared with DM (-) group, respectively.

Among subgroups, most cytokine/chemokine levels including IL-1ra, IL-5, IL-6, eotaxin, G-CSF and IP-10 in group1 were significantly elevated compared with group 4 (Table 4). The serum levels of IL-1β, IL-1ra,

Table 3-a. Association of cytokine/chemokine with hsCRP in WS

Dependent variable	Independent variables	Estimated regression coefficient	S.E.	*p* value
Log (hsCRP)	Intercept	-1.199	1.062	0.268
	Age	0.040	0.019	0.048*
	Sex	0.339	0.389	0.386
	IL-6	0.317	0.132	0.022*

Model (a): log_e(hsCRP) = $a + b1^*$Age + $b2$+Sex + $b3^*$log_e(Cytokine(j)), a was an intercept, $b1^*$, $b2^*$ and $b3^*$ were estimated regression coefficients, and j was an indicator for individual cytokine. Sex was 1 for male and 0 for female. Significance level. *: $p < 0.05$.

Table 3-b. Association of cytokine/chemokine with hsCRP in WS

Dependent variable	Independent variables	Estimated regression coefficient	S.E.	*p* value
hsCRP	Intercept	1.311	0.766	0.097
	Age	0.01	0.014	0.463
	Sex	0.522	0.350	0.146
	IL-12	0.007	0.003	0.029*
hsCRP	Intercept	1.260	0.770	0.112
	Age	0.010	0.014	0.472
	Sex	0.640	0.357	0.082
	VEGF	0.001	0.000	0.018*

Model (b): hsCRP = exp{ $a + b1^*$Age + $b2^*$Sex + $b3^*$Cytokine(j) }, a was an intercept. $b1^*$, $b2^*$, and $b3^*$ were estimated regression coefficients, and j was an indicator for individual cytokine. Sex was 1 for male and 0 for female. Significance level. *: $p < 0.05$

Multiple regression analyses expressed as log_e (hsCRP) = $a + b1^*$Age + $b2^*$Sex + $b3^*$ log_e (cytokine (j)) (model (a)) and hsCRP = exp{$a + b1^*$Age + $b2^*$Sex + $b3^*$ cytokine (j) } (model (b)) were indicated, where a is an estimated intercept, $b1^*$, $b2^*$, and $b3^*$ were estimated regression coefficients and j is an indicator for individual cytokine/chemokine. In these formulae, Sex was 1 for male and 0 in female.

The relationship between IL-6, hsCRP and ageing according to the model(a) was log_e (hsCRP) = -1.199 + 0.04 × Age + 0.339 × Sex + 0.317 × log_e (IL-6) (Table 3-a).

Both IL-12 and VEGF were also significantly associated with hsCRP according to the model (b); hsCRP = exp{ 1.311 + 0.01 × Age + 0.522 × Sex + 0.007 × IL-12} and hsCRP = exp{ 1.26+ 0.01 × Age + 0.64 × Sex + 0.001 × VEGF}, respectively.

IL-5 and IL-13 in group 2 were significantly elevated compared with group 4. Interestingly, MCP-1 level in group1 was significantly decreased compared with group 4. The rest of the cytokines/chemokines levels including IL-2, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10, IL-12, IL-15, IL-17, basic FGF, IFNγ, MIP-1α, MIP-1β, TNFα and VEGF were comparable between subgroups.

4. Discussion

The serum levels of Th1 products (TNFα), Th2 products (IL-4, IL-6, and GM-CSF) and monocyte/macrophage products (IL-15 and MCP-1) were elevated with normal ageing and more elevated in WS. In addition, IL-13 and IP-10 were increased with normal ageing. Ageing-associated elevations of serum IL-13 and serum IL-15 have never been reported in normal human ageing. Although the pro-inflammatory monocyte/macrophage products including IL-1β, IL-1ra and MIP-1αwere decreased with normal ageing, some monocyte/macrophage products (IL-1β, IL-1ra, basic FGF and G-CSF), and Th1 products such as IL-2 and IFNγ, and Th2 cytokine IL-10 were increased in WS.

The WS patients with more inflammatory phenotypes (SU (+) and DM (+)) produced more pro-inflammatory cytokines/chemokines such as IL-1β, IL-1ra, IL-5, IL-6, IL-13, eotaxin, IP-10 and G-CSF than less inflammatory subgroups in WS. Immunological shift to Th2-type T cells was common between normal ageing and WS, although Th1-type cytokines, monocyte/macrophage origin chemokines were also elevated in WS.

One of the characteristic phenotypes in WS is the central obesity with visceral fat deposition (*1*) irrespective of DM and the incidence of central obesity in normal Japanese has increased recently with ageing (*20*).

The WS with inflammatory phenotypes such as SU, DM and central obesity may induce more complicated immunological changes than normal ageing, though both have a common immunological shift to Th2.

CRP is the prototypical acute-phase reactant in man and has been proposed as a marker of atherosclerosis-associated diseases including coronary heart disease and cerebro-vascular accidents (*21-23*). As CRP has an antagonistic pleiotropic activity, CRP induced by IL-6 can act as pro-inflammatory by producing TNF-αand IL-1β (*24*). CRP can also function as a protective machinery by activating the classical pathway of complement system (*25*), enhancing phagocytosis (*26*) and binding to the Fcγreceptors on leukocytes, leading to the anti-inflammatory cytokine IL-10 production and the suppression of IL-12 secretion (*27,28*) as a component of the innate immune system.

Among these cytokines/chemokines, the serum levels of Th2 products (IL-6 and IL-13), IL-15 and

Table 4. Serum cytokines/chemokines in Werner syndrome from different sugroups

Cytokine/chemokine	Subgroups	Mean (pg/mL)	S.E.	*p* value matrix		
				Group 2	Group 3	Group 4
IL-1β	Group 1: SU(+)DM(+) (*n* = 14)	23.1	21.7	0.643	0.645	0.095
	Group 2: SU(+)DM(-) (*n* = 12)	3.8	1.9	-	0.446	0.048*
	Group 3: SU(-)DM(+) (*n* = 4)	1.1	0.3	0.446	-	0.413
	Group 4: SU(-)DM(-) (*n* = 5)	0.8	0.3	0.048*	0.413	-
IL-1ra	Group 1: SU(+)DM(+) (*n* = 14)	87.9	14.6	0.504	0.327	0.008**
	Group 2: SU(+)DM(-) (*n* = 12)	75.1	13.8	-	0.671	0.04*
	Group 3: SU(-)DM(+) (*n* = 4)	66.6	33.6	0.671	-	0.389
	Group 4: SU(-)DM(-) (*n* = 5)	27	10.1	0.04*	0.389	-
IL-5	Group 1: SU(+)DM(+) (*n* = 14)	3.1	0.5	0.742	0.327	0.007**
	Group 2: SU(+)DM(-) (*n* = 12)	2.9	0.5	-	0.684	0.035*
	Group 3: SU(-)DM(+) (*n* = 4)	2.6	1.2	0.684	-	0.286
	Group 4: SU(-)DM(-) (*n* = 5)	1	0.4	0.035*	0.286	-
IL-6	Group 1: SU(+)DM(+) (*n* = 14)	985.7	953.4	0.251	0.798	0.021*
	Group 2: SU(+)DM(-) (*n* = 12)	28	12	-	0.316	0.442
	Group 3: SU(-)DM(+) (*n* = 4)	29	9.2	0.316	-	0.063
	Group 4: SU(-)DM(-) (*n* = 5)	9.8	3	0.442	0.063	-
IL-13	Group 1: SU(+)DM(+) (*n* = 14)	13.6	2.4	0.56	0.878	0.156
	Group 2: SU(+)DM(-) (*n* = 12)	15.7	2.4	-	0.684	0.014*
	Group 3: SU(-)DM(+) (*n* = 4)	18.7	10.9	0.684	-	0.556
	Group 4: SU(-)DM(-) (*n* = 5)	6.7	3.3	0.014*	0.556	-
Eotaxin	Group 1: SU(+)DM(+) (*n* = 14)	137.9	19.7	0.036*	0.035*	0.002**
	Group 2: SU(+)DM(-) (*n* = 12)	61.3	16.5	-	0.77	0.091
	Group 3: SU(-)DM(+) (*n* = 4)	61.9	23.2	0.77	-	0.286
	Group 4: SU(-)DM(-) (*n* = 5)	30.3	13.8	0.091	0.286	-
G-CSF	Group 1: SU(+)DM(+) (*n* = 14)	19.5	2	0.071	0.721	0.014*
	Group 2: SU(+)DM(-) (*n* = 12)	14.9	2.5	-	0.202	0.225
	Group 3: SU(-)DM(+) (*n* = 4)	23.3	6.9	0.202	-	0.063
	Group 4: SU(-)DM(-) (*n* = 5)	8.9	3	0.225	0.063	-
IP-10	Group 1: SU(+)DM(+) (*n* = 14)	3431.6	1220.5	0.322	0.158	0.044*
	Group 2: SU(+)DM(-) (*n* = 12)	1085	206.5	-	0.379	0.195
	Group 3: SU(-)DM(+) (*n* = 4)	783.4	328.8	0.379	-	0.73
	Group 4: SU(-)DM(-) (*n* = 5)	448.6	208.7	0.195	0.73	-
MCP-1	Group 1: SU(+)DM(+) (*n* = 14)	49.1	5.4	0.899	0.878	0.034*
	Group 2: SU(+)DM(-) (*n* = 12)	50.3	7.9	-	1	0.13
	Group 3: SU(-)DM(+) (*n* = 4)	48.6	9.2	1	-	0.19
	Group 4: SU(-)DM(-) (*n* = 5)	75.6	10.4	0.13	0.19	-

Cytokine/chemokine levels among subgroups were estimated by two-sample *t*-test with unequal variances. Significance level: $^{*}p < 0.05$, $^{**}p < 0.01$.

IP-10 were significantly associated with serum level of hsCRP, if age and sex were taken into account in normal ageing (manuscript submitted). However, serum hsCRP was significantly associated with IL-6, IL-12 and VEGF in WS, as indicated in the present study.

IL-4, IL-6 and IL-10 are pro-inflammatory cytokines produced by Th2-type T cells, B cells, classically activated macrophages, adipose- tissue-associated macrophages, fibroblasts and endothelial cells, possibly leading to the activation of wound healing macrophages for tissue repair with fibrosis (*29*) and the abrogation of autophagy and autophagy-mediated killing of intracellular mycobacteria in human macrophages (*5,30*).

Pro-inflammatory chemokine: MCP-1 has been reported to be the products from adipose-tissue-associated macrophages, classically activated macrophages, fibroblasts, endothelial cells and mast cells (*29,31*). Although an increase in the serum levels of IP-10 and MCP-1 with normal ageing has already been described by others (*14,16*), an elevation of serum IL-13 and IL-15 has never been described. IL-13 is aTh2-derived mediator of allergic inflammation and IL-15 is a monocyte/macrophage product from viral infection to proliferate natural killer cells of innate immunity.

These cytokine/chemokine distributions may suggest an association of monocyte/macrophage products-stimulated Th2 type inflammation leading to tissue remodeling and fibrosis by wound healing macrophages in WS and also with normal ageing as suggested by others (*11,29,31,32*).

The elevating inflammation associated with normal ageing may not be the direct result of one-way traffic destruction of tissues, but the sum result of ongoing tissue degradation and repair by a cytokine/chemokine circuit-driven inflammation and regeneration (*33*).

Immunological shift to Th2-type T cells with normal

ageing and WS may stimulate a pro-inflammatory cytokine/chemokine circuit, leading to a systemic chronic inflammation monitored by hsCRP. Monocyte/macrophage products including MCP-1 can be an immunologically possible candidate to stimulate Th2-type T cell shift, though we did not observe a significant association between hsCRP and MCP-1 if age and sex were taken into account.

Further study may be needed to clarify the pathogenesis of Th2 shift and Th2-biased mild inflammation: inflammageing in normal ageing and WS.

In conclusion, minor inflammation-driven inflammageing in WS monitered by hsCRP is associated with increases in IL-6, IL-12 and VEGF.

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Case Report

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Identification of a male with fragile X syndrome through newborn screening

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Summary **A pilot newborn screening (NBS) study for fragile X syndrome was recently conducted at the University of California, Davis Medical Center. The screening study identified a case of a male with the full mutation completely methylated and no detectable expression of the fragile X mental retardation-1 (*FMR1*) gene. The patient was initially seen in clinic at the MIND Institute, for medical follow-up and a genetic counseling session at the chronological age of 3 months. Since then, he has been seen in clinic every six months for follow up, medical examination and developmental assessments. Longitudinally administered developmental testing of the infant has revealed persistent delays in development, consistent with fragile X syndrome. Cascade testing revealed that the patient's mother and two siblings also have the full mutation. The patient has been receiving speech and language therapy, combined with physical and occupational therapies on a weekly basis since the age of one year. He is currently being treated with 2.5 mg of sertraline, which has been demonstrated to be helpful for improving language in young children with the syndrome.**

Keywords: *FMR1* full mutation; trinucleotide repeat diseases; genetic counseling; cascade testing

1. Introduction

Fragile X syndrome (FXS) is the most common cause of inherited intellectual disabilities (ID), and is due to the expansion of a trinucleotide CGG repeat in the promoter region of the *FMR1* gene. Individuals with alleles harboring 200 or more CGG repeats are usually subject to hypermethylation of the *FMR1* gene, which impairs the production of *FMR1* protein (FMRP) and causes the abnormal neural development and subsequent intellectual disability (ID) typical of FXS. FXS affects as many as 1 in 5,000 males (*1*) and autism spectrum disorder (ASD) may be present in as many as 60% of these individuals (*2-4*). Although the parental first concern occurs usually at 12 months, the typical age at diagnosis is approximately 35-37 months (*5*) and in some countries can occur much later, particularly for females with FXS.

Newborn screening for *FMR1* mutation is not mandated in any state in the US, and, until recently, testing was costly and therefore not available for large population screening. In addition, until recently, the paucity of targeted treatments for fragile X-associated disorders and the lack of data on early intervention has diverted the attention and augmented the controversy. Population screening for fragile X mutations and, particularly newborn screening, has therefore been the object of ongoing controversy particularly regarding to the value of patients' discovery of their genetic status, at birth for the newborn and for other family members (*6,7*). There is also concern for identifying a carrier at birth since the premutation is associated with a neurodegenerative disorder, the fragile X-associated tremor ataxia syndrome (FXTAS) in aging for which currently, the diagnosis is not predictable. However, the premutation is also associated in some carriers with developmental problems that can benefit from early intervention (*8,9*).

In the past few years, significant advances in genomic testing, have improved the methods for

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detection of *FMR1* mutations, resulting in more accurate testing with low cost and timely return of test results and, have led to several large population screening studies (*10,11*). In addition, ongoing research, pursuing targeted treatment for fragile X-associated disorders, has revealed potential psychopharmacologic and educational interventions (*12-18*). Though screening and detection of *FMR1* mutations in the newborn period is not yet fully embraced (*19*), many parents took advantage of the opportunity to screen their newborn when made available as part of a voluntary screening during the state mandatory testing (*20*). Indeed two recent pilot newborn screening studies reported a high acceptance rate of > 70% (*11,21*) indicating that universal newborn screening for FXS may be more widely accepted and advisable than previously believed.

In this study, we report on the identification of a male with a *FMR1* full mutation through a pilot study of NBS conducted at the UC Davis Medical Investigation for Neurodevelopmental Disorders (MIND) Institute (*11,22*) and we provide a summary of the clinical involvement during the first 3 years of his life.

2. Newborn screening for FXS

Each year, roughly 1,700 babies are born in the Pediatric units at the University of California Davis Medical Center (UCDMC). A pilot NBS study for FXS was conducted using blood spots at the UCDMC, approved by the Institutional Review Board (IRB), as previously described (*11,23*). Parents of the newborns were approached by research assistants who explained the research and carried out the process of informed consent, during their stay on the Labor and Delivery units. According to the Institutional Review Board (IRB) approved protocol, a blood sample was obtained on individual filter paper (FTA or 903 blood spot cards, Whatman) and polymerase chain reaction (PCR) was performed to determine the CGG repeat size as previously described (*11*).

If a repeat size in either the premutation range (between 55 and 200 CGG repeats) or full mutation range (> 200 CGG repeats) was discovered, the family was contacted by the genetics counselor at the MIND Institute after the infant reached 2 months of age. The genetic counselor also invited the family of the newborn to the MIND Institute for an appointment to provide confirmatory genetic testing, genetic counseling for the family, a medical examination, and developmental assessments for the infant. Family members who wished to participate in further research, tracking the development of children with fragile X gene mutations were scheduled to visit at six-month intervals for medical exams and developmental assessments at no cost (*11,23*).

PCR on blood spot was performed as previously described (*11*). Genomic deoxyribonucleic acid (DNA) was isolated from 3 ml of peripheral blood lymphocytes using standard methodology (Qiagen, Valencia, CA). Repeat size and methylation status were determined using PCR and Southern blot analyses using the *FMR1*-specific probe StB12.3. as described in previous studies (*24,25*). *FMR1* mRNA expression levels were measured by quantitative reverse transcription-PCR (qRT-PCR) as described (*26*).

3. Case report

3.1. *Clinical history*

The identified newborn was born vaginally at 38 weeks 6 days gestation and his birth weight was 7lb 8oz, his Apgars were 7 and 9. Meconium was present at birth. His mother had gestational diabetes mellitus and received insulin during the last four months of pregnancy.

The patient demonstrated restless and fitful sleep as an infant, in addition to significant irritability and tantrums. Throughout development he has been very interested in social interaction. He can be hyperactive and also perseverative in his behavior and language. His developmental milestones included sitting at ten months, crawling by 11 months, and cruising at one year, but he was not walking independently until 18 months. He began using single words at two years of age. He was referred for early intervention in the first year of life and he received speech and language therapy and physical therapy (PT) and occupational therapy (OT), which included sensory integration, on a weekly basis.

He demonstrates poor eye contact; also must lean his head back in order to see what is in front of him due to his congenital bilateral ptosis. A developmental pediatrician recommended surgical correction at a later date.

The patient has been seen in clinic at the MIND Institute, for medical examination and developmental assessments, beginning at 3 months of ages. On his first examination (at 3 months) the patient presented with a hydrocele on the right testicle, which resolved spontaneously. At 6 months his head circumference was 42.5 cm, at 12 months it was 45 cm (97th percentile) and at 36 months it was 48 cm (50th percentile).

Developmental assessments show global delays in various domains of ability. The Mullen Scales of Early Learning (MSEL) were administered at 6, 12, 24, and 30 months of age, as were the Vineland Adaptive Behavior Scales (Table 1 and Table 2). At 27 months he was evaluated for autism spectrum disorder (ASD) using the Autism Diagnostic Observation Schedule (Module I, some words). His Social Affect and Restricted and Repetitive Behavior Total was 11 (cut off for autism is 12, for autism spectrum, 8). His intervention intensified with Applied Behavioral Analysis (ABA) therapy, administered at preschool, because of his ASD diagnosis.

The Preschool Language Scales evaluation placed

Table 1. Vineland Adaptive Behavior Scales (VABS)

Age (months)	Communication	Daily Living Skills	Socialization	Motor Skills	ABC
6	74	67	66	61	64
12	84	88	73	65	74
24	90	96	92	90	90
30	84	82	91	90	84

Table 2. Mullen Scales of Early Learning (MSEL)

Age (months)	Gross Motor Skills	Visual Reception	Fine Motor Skills	Receptive Language	Expressive Language	ELC
6	3	4	2	4	3	63
12	10	8	6	9	6	60
24	16	16	18	16	14	63
30	16	19	21	24	20	60

Table 3. Molecular and clinical measures

Case	Age (years)	Gender	Category	FSIQ	IQ test	CGG size	AR	*FMR1* mRNA (Std. Err)
Proband	3	Male	FM	62	Binet	390, 460, 780, 1130	N/A	0.01 (0.05)
Mother	36	Female	FM	103	WASI	28, 270, 400, 570, 650	0.88	1.4 (0.02)
Brother	4	Male	FM	49	Mullen	223, 389, 532, 1000	N/A	0
Sister	14	Female	FM	65	Binet	33, 431, 561, 1093	0.77	1.46 (0.15)

FM= full mutation; AR= Activation Ratio (percent of cells carrying the normal allele on the active X chromosome).

him at a 21 months developmental level although he was 32 months at time of testing. He was entered into a controlled trial of sertraline for young children with FXS at 2 years of age but he was later found to have randomized to placebo so he was subsequently treated with 2.5 mg of sertraline since this has been shown to be helpful in young children with FXS (*27*). The patient is also currently taking a multivitamin and vitamin D.

Genetic counseling and cascade testing were carried out for the family members. The mother and the additional 2 siblings were found to also have the full mutation. The sister was enrolled into a study of an metabotropic glutamate receptor 5 (mGluR 5) antagonist and the brother was enrolled in a clinical trial using ganaxolone (*16*).

3.2. *Molecular measures*

DNA molecular testing results on the newborn and his immediate family members, including the mother and two siblings (one male and one female). DNA was isolated from 3ml of blood using standard protocol (Qiagen, Valencia, CA). Using Southern Blot and PCR analysis (*24,25*) the presence of a full mutation was detected in all of them as depicted in Figure 1. CGG repeat number, Activation Ratio, methylation and *FMR1* mRNA levels are reported in Table 3.

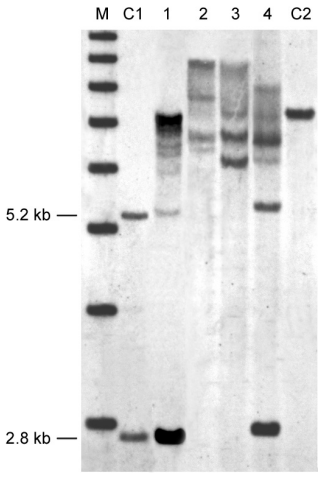

Figure 1. Southern blot analysis of genomic DNA isolated from a normal control female (C1), probands' mother (Lane 1), male proband (Lane 2), proband's brother (Lane 3), proband's sister (Lane 4) and from a full mutation control (C2). M= DNA marker, 1 kb ladder. Normal unmethylated band (2.8 kb) and normal methylated band (5.2 kb) shown on the left. (C1) and a full mutation control sample is shown on the right (C2). Southern blot analysis was carried out on 7-10ug of with Eco RI and Nru I restriction enzymes. Fragments were separated on an agarose gel, transferred on a nylon the membranes, which were hybridized with a FMR1-specific genomic probe, StB12.3. Additional details of the method are as described in (*25*).

4. Discussion

The identification of a child with FXS at the time of birth can lead to earlier intervention, which can be beneficial for the development of the child (*27,28*). This case began intervention before the end of the first year and the mother benefitted from cascade testing, demonstrating how testing can be beneficial not only for the proband, but for the family as well. The mother is a single parent and she is raising her 3 children

without much family support. Thus, the relationship that she established with the MIND Institute has been helpful and supportive to her and her family.

This child has done well with interventions. The cause of his congenital ptosis may be related to his FXS, although he has a more severe ptosis than what has been previously reported (*29*). Although his visuo-spacial development would have likely improved with earlier surgery, it was thought to be of high risk. Children under 5 years old with ASD have been documented to have low serotonin levels in the frontal regions of the brain by positron emission tomography (PET) imaging (*30,31*). The newborn has had the advantage of an early intervention treatment trial of sertraline which enhances serotonin and stimulates neurogenesis and Brain Derived Neurotropic Factor (BDNF) in the CNS (*32*). In a previous retrospective study of sertraline in young children with FXS those who received treatment with sertraline had higher receptive and expressive language than those who did not receive sertraline (*27*). Additional treatments are available to the newborn and his family. Because early treatment with minocycline, which lower the elevated matrix metallopeptidase 9 (MMP-9), levels observed FXS and has shown efficacy in children with FXS (*15*), he will also undergo a trial of minocycline in the near future in addition to sertraline. Mother has also treated him with antioxidants and infant massage therapy.

Newborn screening can lead to cascade testing and the identification of other family members with an *FMR1* mutation can be beneficial information to other family members (*23*). Although young premutation carriers can be identified who may develop medical problems such as FXTAS or fragile X-associated primary ovarian insufficiency (FXPOI) with age, they can be followed closely for developmental problems that they might demonstrate and subsequently benefit from treatment. In addition, there are a number of interventions that may help to avoid the aging problems of some carriers and such interventions have been reviewed recently (*33,34*).

In conclusion, we have identified a newborn with FXS through newborn screening. To our knowledge this is the first case of a full mutation with FXS identified through newborn screening reported in the literature.

Acknowledgements

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Case Report

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An isolated single L-II type coronary artery anomaly: A rare coronary anomaly

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Summary **The incidence of congenital artery anomalies is 0.2-1.4%, and most are benign. Single coronary artery (SCA) anomalies are very rare. The right coronary artery (RCA) originating from the left coronary system is one such SCA anomaly, and the risk of sudden cardiac death (SCD) increases if it courses between the pulmonary artery and aorta and coexists with other congenital heart diseases. Additionally, coursing of the RCA between the great vessels increases the risk of atherosclerosis. We herein present the case of a 57 year-old man who was admitted to our cardiology outpatient clinic and diagnosed with an SCA anomaly in which the RCA arose from the left main coronary artery (LMCA) and coursed between the pulmonary artery and aorta. However a critical stenosis was not detected in imaging techniques, and myocardial perfusion scintigraphic evidence of ischaemia was found in a small area. Therefore, he was managed with conservative medical therapy.**

Keywords: Coronary vessel anomalies, coronary angiography, multidetector computed tomography

1. Introduction

Although most coronary artery anomalies are benign and are detected incidentally during diagnostic angiography, some anomalies result in catastrophic clinical outcomes such as sudden cardiac death (SCD) (*1*). Therefore, these anomalies are among the most complex and significant subjects in the field of cardiology. An anatomically correct definition of these anomalies is important to predict complications that may develop during myocardial revascularisation. We herein present a case involving a patient with a single coronary artery (SCA) anomaly in which the right coronary artery (RCA) arose from the left main coronary artery (LMCA) and coursed between the pulmonary artery and aorta.

2. Case report

A 57 year-old male was admitted to our outpatient clinic because of a 3-month history of exertional dyspnoea. He had no known history of coronary artery disease (CAD) or systemic disease. This patient's cardiovascular risk factors included a smoking habit (20 pack-years) and older age. Physical examination revealed a blood pressure of 120/80 mmHg and heart rate of 78 bpm. Increased bronchovascular branching was noted on telecardiography, and electrocardiography revealed a normal sinus rhythm. His echocardiographic findings were normal. A 1-mm ST depression was observed in the inferior leads during a treadmill exercise test; however, the patient did not have typical chest pain accompanying the electrocardiographic changes. He underwent coronary angiography (CAG) with a prediagnosis of CAD after he had been evaluated at a pulmonology clinic. The LMCA was selectively cannulated with a JL4-6F diagnostic catheter (Diagnostic catheter, Medtronic, New York, USA). No atherosclerotic lesions were observed on CAG; however, an SCA anomaly was seen in which the RCA originated from the LMCA (Figure 1). Additionally, the RCA coursed between the aorta and pulmonary artery, and a critical stenosis was not detected (Figure 2). Multislice computed tomography (CT) angiography was performed after the patient was discharged 1 week later on account of the fact that it can assist delineating the proximal course of the artery and

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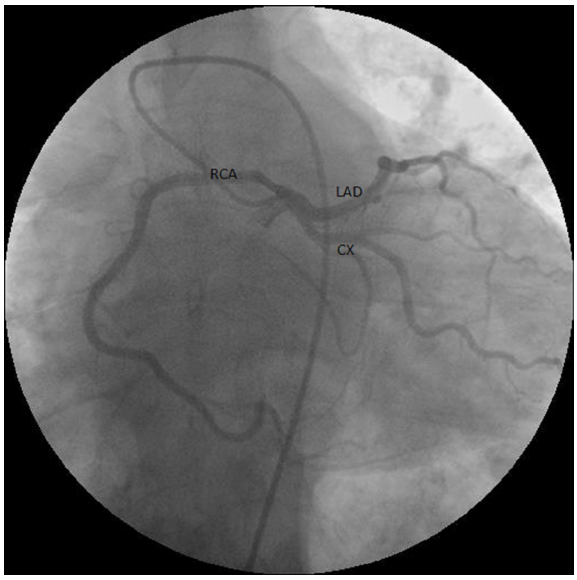

Figure 1. Coronary angiography revealed an SCA anomaly in which the RCA originated from the left main coronary artery (LMCA). SCA, single coronary artery; RCA, right coronary artery.

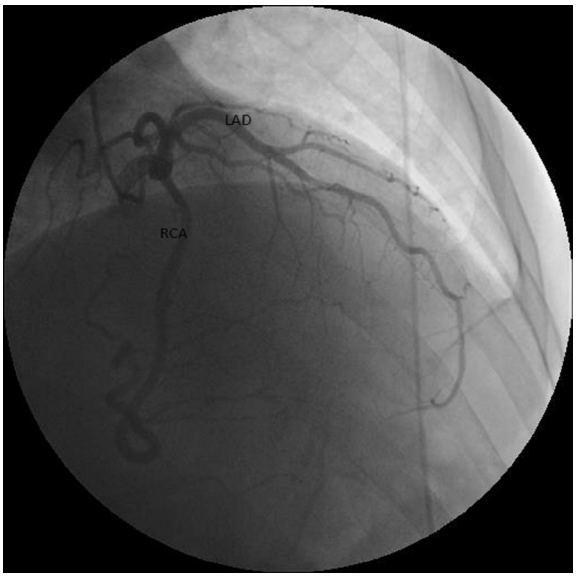

Figure 2. Coronary angiography revealed that the RCA coursed between the aorta and pulmonary artery.

provides excellent high-quality images. Thus, the origin and course of the RCA were able to be better evaluated with three-dimensional imaging (Figure 3). Resting and stress Tc-99m tetrofosmin single photon emission CT (SPECT) revealed 7% ischaemia in the RCA vascular territory. In addition, no other congenital cardiac defects accompanying the coronary anomaly were seen in our patient. Therefore, he was managed with conservative treatment comprising a betablocker and isosorbide mononitrate therapy.

3. Discussion

Congenital coronary artery anomalies are seen in 0.2-

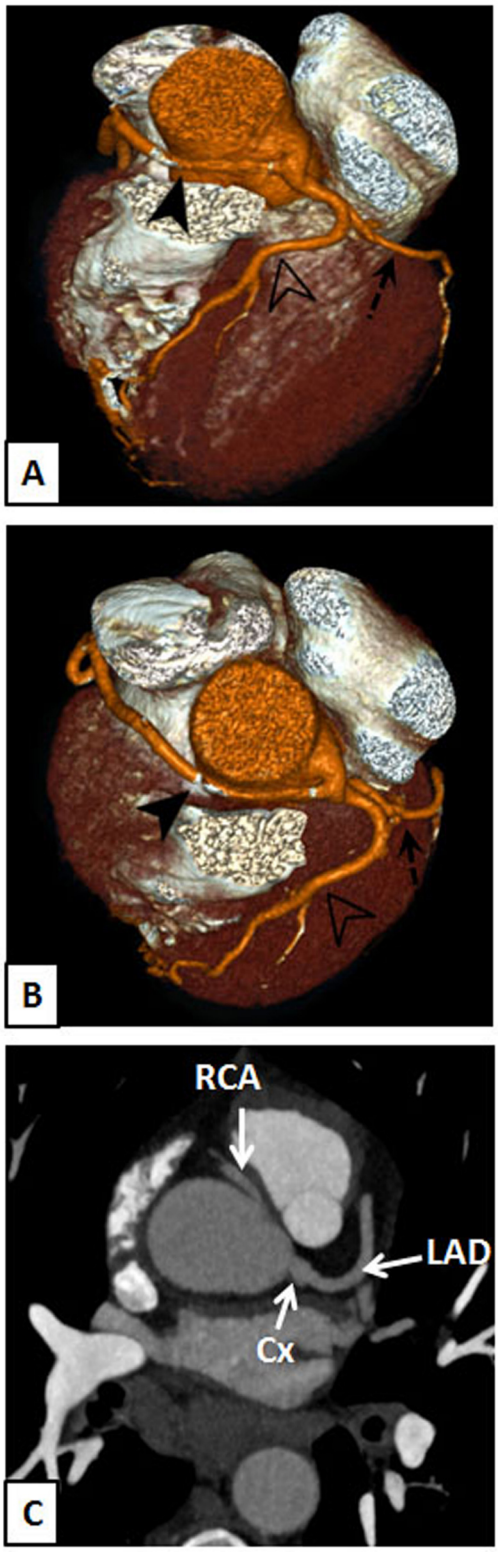

Figure 3. A three-dimensional volume-rendering image (A, B) and multiplanar reconstruction axial image (C) showing the SCA anomaly. RCA, black arrow head; LAD, white arrow head; CXA, dashed arrow.

1.4% of the normal population (*2*). Lipton *et al*. (*3*) reported that an SCA originating from the right or left coronary system constituted 3.3% of all congenital coronary anomalies in their study. The classification of SCA anomalies have been made by some authors.

For instance, in 1950, Smith described three different types of SCA (*4*). Recognizing the deficiency of such broad categories, Ogden and Goodyer in 1976 proposed a more complete classification (*5*). They classified the SCA into 14 basic distribution patterns. Yamanaka and Hobbs in 1990 modified the classification previously described by Lipton *et al*. (*6*). Based on the modified Lipton classification, each anomaly is coded with an R or L depending on the localisation of the sinus from which the coronary anomaly originates. Additionally, the anomalies are classified into three types according to the anatomical distribution on the ventricular surface: type I = the vessel follows the course of normal left or right coronary artery with a continuation into the missing artery's territory; type II = an anomalous artery arises from the proximal part of the other normal artery and courses the base of the heart before taking the native course; and type III = LAD and CX arteries originating from the proximal part of the RCA. The third component of the Lipton classification associated with the course of the transfer branch: the aberrant artery could take one of several different pathways to reach its vascular territory. These pathways are indicated as: type A (anterior to the right ventricular outflow tract); type B (between the aorta and pulmonary trunk); type P (posterior to the great vessels); type S (above the interventricular septum); type C (combined type). The present case can be classified as an isolated single L-II B subtype coronary artery anomaly.

Furthermore, RCA anomalies have many origins; they may originate from the left or posterior sinus of Valsalva, ascending aorta, pulmonary artery, LMCA, LAD, or CXA. The incidence of SCA anomalies involving the RCA originating from the left coronary system (L-I and L-II) was reported as 0.016-0.090% in the study by Lipton *et al*. (*3*). This incidence was reported as 0.036% among 16,573 patients included in the retrospective CAG screening study by Yuksel *et al*. (*7*). In the literature, most RCAs originated from the proximal or mid-LAD; RCA anomalies originating from the left coronary cusp or LMCA have been rarely reported.

SCA anomalies may coexist with other congenital heart diseases, mainly transposition of the great vessels followed by coronary arteriovenous fistula, bicuspid aortic valve, tetralogy of Fallot, truncus arteriosus, ventricular septal defect, patent ductus arteriosus, and patent foramen ovale (*8*). SCA anomalies may appear incidentally on CAG during screening and are known to increase the likelihood of the coexistence of congenital cardiac defects. No other congenital cardiac defects accompanying the coronary anomaly were seen in our patient.

Most patients are asymptomatic and have a benign clinical course. Nevertheless, an increased incidence of atherosclerosis is observed among patients with SCA anomalies although the relationship between atherosclerosis and SCA anomalies is not definitive. A few potential mechanisms have been proposed to explain atherosclerosis. Abnormal origin, long traveling distance, intramural course of the aberrant artery and particularly compression between the great vessels may precipitate endothelial injury and atherosclerosis. Atherosclerosis requiring medical therapy or percutaneous or surgical revascularisation has been seen in approximately half of the reported cases to date (*9*). This finding seems to support the theory that the risk of atherosclerosis is higher in patients with than without SCA anomalies. Symptoms including chest pain, dyspnoea, palpitation, syncope as in CAD, and myocardial infarction and SCD may be seen due to myocardial ischaemia. In patients without atherosclerosis, the development of ischaemia may be explained by the stenotic slit-like orifice, acute angle take-off, coronary vasospasm or compression of great vessels. If the RCA courses between the pulmonary artery and aorta and is under mechanical compression, coronary perfusion decreases. As a result, increased great vessel dilation leads to myocardial ischaemia, particularly during exercise, and arrhythmia and SCD may occur. Taylor *et al*. (*10*) investigated the association between SCD and congenital coronary artery anomalies. They reported that the incidence of exercise-related SCD was significantly higher in a nonatherosclerotic young population with an abnormal RCA originating from the left coronary system and coursing between the pulmonary artery and aorta. Our patient had an SCA anomaly with specific cardiovascular risk factors; however, his CAD was nonatherosclerotic and his RCA coursed between the great vessels. 7% ischaemia in the RCA vascular territory was seen on SPECT examination due to extrinsic compression. Therefore a surgical procedure was not offered to treat, and he was managed with conservative medical therapy.

Although cardiac catheterisation is the gold standard for the identification of coronary anomalies, coronary CT angiography is a useful noninvasive method for evaluating the course of these abnormal coronary arteries, verifying the diagnostic accuracy, and determining the optimal treatment. The excellent spatial resolution of coronary CT angiography makes this technique very suitable to detect the relationship of the anomalous vessels with the aorta, pulmonary artery and cardiac structures (*11,12*). Besides, cardiac magnetic resonance imaging (MRI) is a convenient tecnique to determine coronary anomalies and it may be superior to conventional angiography, particularly in patients with congenital heart defects. However, due to low spatial resolution, this imaging technique is already less helpful in evaluating the distal coronary system (*13,14*). Therefore, we diagnosed the SCA anomaly by cardiac catheterisation and further preferred coronary CT angiography to delineate the course of the aberrant artery in relation to the great vessels.

4. Conclusion

An SCA anomaly involving the RCA originating from the left coronary system is an important risk factor for SCD if the RCA courses between the great vessels and a critical stenosis is detected. However, in our case a critical stenosis was not detected in imaging techniques. Therefore conservative medical therapy was preferred. While cardiac catheterisation is the gold standard for the identification of coronary anomalies, coronary CT angiography is a useful noninvasive imaging technique and plays an important role for the diagnosis of such anomalies.

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Case Report

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Azathioprine-induced atrial fibrillation

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Summary **Azathioprine, a purine analogue that competitively inhibits the biosynthesis of purine nucleotides, is used in a wide range of conditions. Although its side-effects are well known, cardiac side effects like paroxysmal atrial fibrillation (AF) are based on only a few case reports. We describe here the case of a 55-year-old woman with primary biliary cirrhosis who presented a first-detected, symptomatic AF 2 h after azathioprine therapy which resolved after discontinuation of the drug with no predisposing factors for supraventricular arrhythmias (systemic hypertension, diabetes or coronary artery disease). The temporal coincidence of atrial fibrillation and azathioprine intake and disappearance of the AF episode after discontinuation of therapy allows us to suggest an intrinsic pro-arrhythmic effect of azathioprine. Therefore, physicians should be aware of this problem when this drug is administered.**

Keywords: Azathioprine, atrial fibrillation, cardiac side effects

1. Introduction

Azathioprine, a purine analogue that competitively inhibits the biosynthesis of purine nucleotides, is used in a wide range of conditions such as inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus, solid organ transplantation and vasculitis. Azathioprine is quickly and nearly completely absorbed from the digestive tract. Bioavailability varies greatly between individual patients, between 30 and 90%, because the drug is partly inactivated in the liver. The peak serum levels occur roughly 2 h after ingestion, and the average half-life is 26 to 80 min for azathioprine and 3 to 5 h for drug plus metabolites. Azathioprine is extensively metabolized, and only about 2% is excreted, unchanged, in the urine, 20-30% is bound to plasma proteins while circulating in the bloodstream. The side effects of azathioprine are well-documented and include dose dependent myelosuppression and hepatotoxicity as well as a dose-independent hypersensitivity syndrome ranging from isolated fever, and rash to multi-organ failure, which is relatively less frequent (*1*). Cardiovascular side effects have included rare cases of hypotension, including cardiogenic shock (*2*). A few cases of atrial fibrillation (AF) induced by this drug have been reported (*3,4*) although causality is unknown. We report a case of a 55-year-old woman with primary biliary cirrhosis who developed a first-detected, symptomatic AF after azathioprine therapy.

2. Case repor

A 55-year-old woman presented to the emergency department complaining of palpitation lasting for 4 h, which began 2 h after 50 mg of azathioprine therapy. Her body weight was 75 kg and her height was 170 cm. Physical examination revealed a blood pressure of 130/80 mmHg, clear lungs and normal heart sounds. The temperature was 36.4°C. There was no history of fever, illicit drugs, alcohol or exposure to toxic chemicals. Electrocardiographic (ECG) examination showed atrial fibrilation of 130 beats/min without conduction abnormalities or ST-T changes (Figure 1) Trans-thoracic echocardiography showed normal left ventricular size and function with no valvular abnormalities and normal left atrial para-sternal diameter of 39 mm and left ventricular ejection fraction of 65%. Chest radiograph and routine laboratories including cardiac enzymes were

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normal. Hematological examination, urinary analysis and thyroid function were all normal. Her medical history included primary biliary cirrhosis for 2 years. The patient's medications included ursodeoxycholic acid, and prednisolone and had not changed for many months except for the recent addition of azathioprine 2 h before her arrival at the emergency department. She reported that her complaints started immediately after azathioprine therapy which was the first dose given for prımary biliary cirrhosis. Metoprolol *i.v*. was administered immediately with resultant conversion to normal sinus rhythm within 1.5 h (Figure 2). Patient was discharged without antiarrhythmic medication. The therapy with azathioprine was discontinued in view of the suspicion of its pro-arrhythmic effect. After stopping azathioprine the patient's condition markedly improved. A 24 h ambulatory ECG monitoring revealed no cardiac arrhythmias as well as during the control examination one month after the attack of AF. No other episodes were reported and the patient was asymptomatic without medication after 3 months of follow-up. Other treatments were not changed.

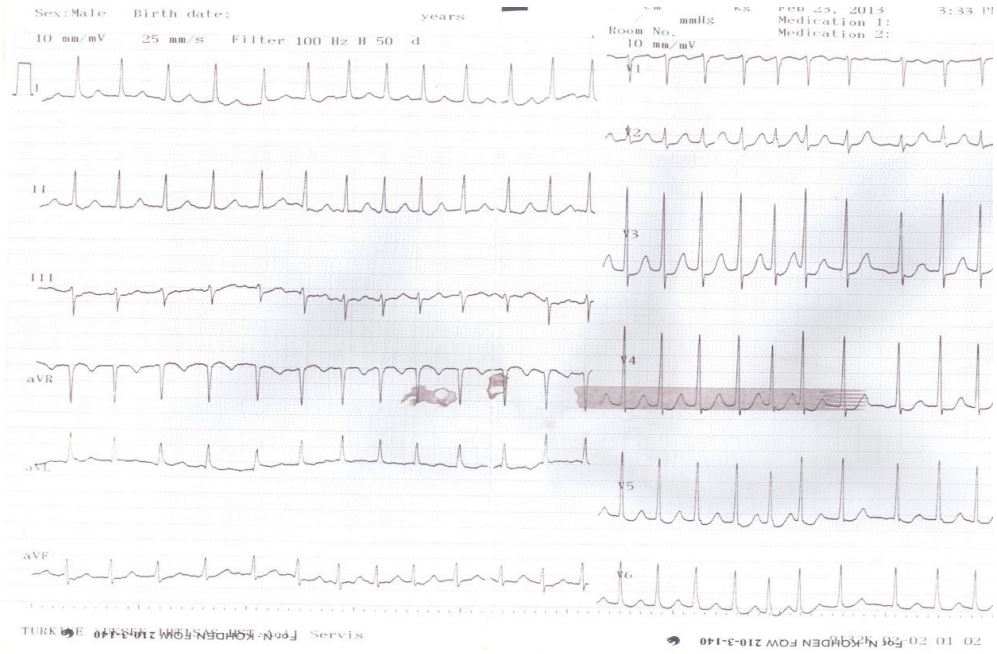

Figure 1. Examples of ECG showing that AF developed after azathioprine intake. ECG, electrocardiographic; AF, atrial fibrillation.

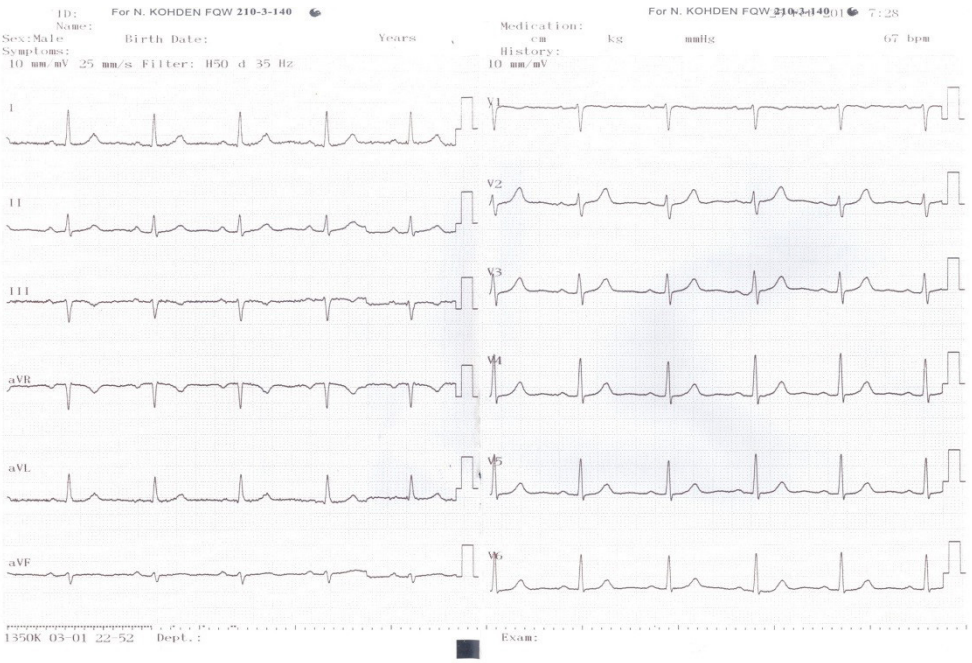

Figure 2. Complete restoration to sinus rhythm using IV metoprolol after discontinuation of Azothiophirin.

3. Discussion

AF is the most common arrhythmia treated in clinical practice and the most common arrhythmia for which patients are hospitalized. AF is associated with risk of stroke, and all-cause mortality and development of heart failure. Acute temporary causes of AF include alcohol abuse, excessive coffee intake, surgery, pericarditis, myocarditis, and pulmonary embolism. Medications also have been associated with the induction of AF both in case reports and clinical trials (*5*). This is one report of a first-detected, symptomatic AF probably due to cardiac toxicity of azathioprine.

A pro-arrhythmic effect of azathioprine is strongly suggested by the temporal coincidence of atrial fibrilation and azathioprine intake in our patient with no cardiovascular diseases. The AF episode was closely related in time to treatment with azathioprine that occurred 2 h after drug intake. We couldn't determine any other precipitating factor beyond the azathioprine therapy as a cause for the arrhythmia. The complete symptoms resolution and the disappearance of AF episodes after azathioprine discontinuation while other drugs were continued is in line with its pharmacokinetic. Since average plasma half-life is 26 to 80 min for azathioprine the precipitating role of this drug is strongly suspected because arrhythmic episodes occurred 2 h after azathioprine and no other predisposing clinical factors, such as electrolyte imbalance, neuro-autonomic dysfunction, thyrotoxicosis or subclinical hyperthyroidism, pulmonary embolism, hypertensive crisis, alcohol abuse, or excessive coffee intake were involved. Although several case reports and case-control studies have associated this condition with the use of systemic corticosteroids, azathioprine seems more likely as a cause for the arrhythmia since no episodes occurred when this drug was discontinued and the other drugs such as prednisolone used in our patient were continued.

We have found three other similar reports of pro-arrhythmic cardiac toxicity like AF during azathioprine use. A case of fast AF induced by treatment of psoriasis with azathioprine had been reported (*3*). However alcohol consumption and fever could be the possible trigger of atrial fibrilation in that case. In another case in ulcerative colitis Cassinotti *et al*. reported very rapid appearance of AF after 2 h of drug administration as in our case (*4*). Other cardiac effects described in the literature are hypotension, tachycardia and some forms of shock in the context of hypersensitivity reactions occurring within 4 weeks of starting azathioprine (*2*).

Mechanism of arrhythmia seen during treatment with azathioprine is unknown. All drug induced AF is reported to have the following main mechanisms: adrenergic or vagal stimulation, direct cardiotoxicity, changing atrial conduction, refractoriness or automaticity, coronary vasoconstriction/ischemia, and electrolyte disturbances (*5*). In the literature there have been some reports focused on effects of azathioprine on ion channels which may be the cause of cardiac rhythm disturbance (*6,7*). But they failed to show its electrophysiological effects by modulating ionic transport across cellular membranes. The pathophysiology of rhythm dysfunction during treatment with azathioprine by the way mentioned above remains to be established.

This case highlights an unusual causal relationship between azathioprine and AF. We conclude that AF is an unusual, but potentially dangerous, side-effect of azathioprine therapy. The arrhythmia should be suspected whenever patients complain of dyspnea and palpitations beginning immediately after treatment. In these cases, the treatment for AF consists of anti-arrhythmic drugs in order to obtain a sinus rhythm or control the heart rate. It is important for physicians using azathioprine to keep in mind this serious but reversible adverse effect.

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Case Report

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Infantile systemic hyalinosis in identical twins

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Summary **Infantile systemic hyalinosis (ISH) is a rare disorder belonging to the heterogeneous group of genetic fibromatoses. It is a rare, progressive, fatal autosomal recessive condition characterized by widespread deposition of hyaline material in many tissues caused by mutations in the anthrax toxin receptor 2 gene - ANTXR2. It presents hyperpigmented skin over bony prominences. Characteristic purplish patches develop over the medial and lateral malleoli of the ankles, the metacarpophalangeal joints, spine and elbows, with progressive joint contractures, osteopenia, skin abnormalities and chronic severe pain. The present case reports the occurrence of infantile systemic hyalinosis in twin brothers five months of age who had come for early intervention for joint contractures representing characteristic brownish patches over bony prominences. ISH cases reported until this date have been less than 20 and the present case is unique in nature since this is the first time ISH is reported in twins globally and the symptoms have been identified at an early age.**

Keywords: Infantile systemic hyalinosis, Hyalinosis, Mutation in ANTRX2

1. Introduction

Infantile systemic hyalinosis (ISH) is a rare disorder of genetic fibromatoses which is a fatal, autosomal recessive disorder with deposition of hyaline material in many tissues (*1*). ISH (severe form) is a part of hyaline fibromatosis syndrome and must be differentiated from juvenile hyaline fibromatosis (mild) as both belong to hyaline fibromatosis syndrome and recent data indicate that both severe and mild forms of inherited systemic hyalinosis are caused by mutations in ANTXR2/CMG 2 (capillary morphogenesis gene 2) (*2-4*).

Infantile systemic hyalinosis presents hyperpigmented skin over bony prominences, characteristic purplish patches develop over the medial and lateral malleoli of the ankles, the metacarpophalangeal joints, spine and elbows (*5*), with progressive joint contractures, osteopenia, skin abnormalities, chronic severe pain and widespread deposition of hyaline material in many tissues such as the skin, skeletal muscle, cardiac muscle, gastrointestinal tract, lymph nodes, spleen, thyroid and adrenal glands (*6,7*).

Clinical features are evident either at birth or within the first six months of life. Small pearly papules (predominantly on the face, scalp, and neck), massive gingival hypertrophy, and fleshy nodules in the perianal region are the dermal lesions found in ISH (*2,3*). Excessive crying and severe pain on passive movement is common. A depressed nasal bridge, variable ear malformations and a slightly coarse facial appearance may be present. Death occurs secondary to sepsis with renal, respiratory and heart failure, usually by the age of two years due to intractable diarrhea (*7-9*). The survival age may vary from 2-6 years based on management with nutritional supplementation, physiotherapy for joint contracture, use of NSAIDS and oral rehydration therapy and antibiotics for diarrhea.

2. Case Report

The present case is about identical twins 5 month old baby boys, who have come for early intervention and treatment for relieving joint contractures (Figure 1A). When they were two months old, they were confirmed as victims of infantile systemic hyalinosis by a genetician. They were offspring of third degree

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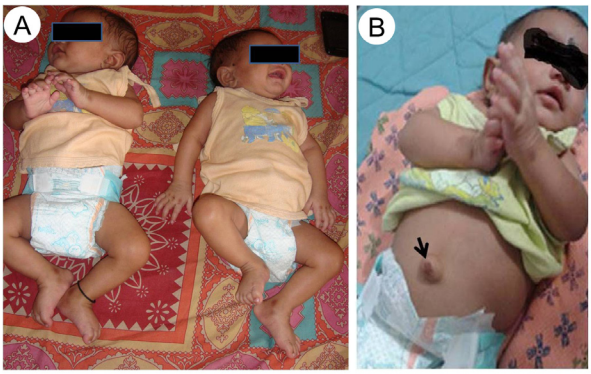

Figure 1. Image of infantile hyalinosis in identical twins. (A) showing the contractures at knee joints; **(B)** showing umbilical hernia in one of the twins (Arrow mark).

Figure 2. Image of infantile hyalinosis in identical twins. Showing the purplish patches over metacarpophalangeal joints characteristic of hyalinosis.

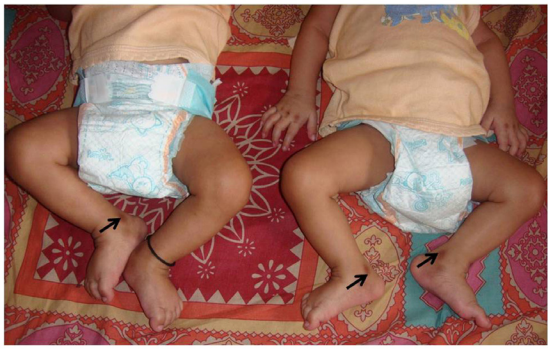

Figure 3. Image of infantile hyalinosis in identical twins. Showing purplish patches development over the medial malleoli (Arrow marks) of the ankles.

parents whose grand fathers are brothers. The mother of the children who is of 21 years old conceived at 19 years and her first pregnancy was medically terminated in her third month because of detection of congenital malformations identified through ultrasonography. After one and half years she delivered full term monozygotic twin baby boys, sharing a single placenta, by caesarian section. Mother was diagnosed as hypothyroid earlier and she was on medication for this condition.

One of the baby boys was 200 grams less in weight at birth, weak when compared to the other and later maintained a 500 gram difference in weight. The parents on detailed enquiry informed us that both baby boys started crying continuously and had disturbed sleep. When they try to lift them or during bathing or dressing, the twins were crying and the one who is weak had more problems at the shoulder girdle. Both of them had a mild umbilical hernia, whenever they cried the loop herniated prominently (Figure 1B). By the second month parents noticed joint contractures and the boys were treated with vitamin D with no success. They presented typical flexion at elbow and extension at wrist and arms in a pronated position. They failed to do supination even on trials. Later they consulted a genetician who diagnosed them as ISH by presence of joint contractures and purplish patches over malleoli and wrist. Characteristic purplish patches increased over the medial and lateral malleoli of the ankles and developed over the metacarpophalangeal joints by the third month (Figures 2 and 3). Contractures were progressive and extremities became fixed with the hips and knees in flexed position as indicated in radiographs and the ankles in dorsiflexion. Both developed diarrhea of unknown etiology by five months for ten days and were treated with antibiotics and oral rehydration therapy by a pediatrician. By the sixth month they developed reddishness on the face whenever they cried and also suffered from recurrent diarrhea. The baby boy with less weight started developing fleshy nodules in the perianal region as well as on the face by 8 months, whereas the elder one had nodules which appeared first on the face by 8 months and perianal region by 11 months. Craniofacial dysmorphism exhibited by ISH was not observed in them. Gingival hypertrophy characteristic of ISH appeared in them by 11 months of age (Figure 4 A-F). They presented with delayed developmental milestones in motor activities as well as in speech. Both babies have continuous recurrence of diarrhea. Polymerase chain reaction and Sanger sequencing covering all exon – intron boundaries of the *ANTXR2* gene was carried out in blood samples to find the mutation. BLAST analysis was done to check for any pathogenic variation. This test revealed the sample is homozygous for insertion mutation c.277_278insATTATTT (or p.L93Yfs*14) in exon 3, leading to premature termination of protein.

3. Discussion

The present case of identical twins exhibits all the characteristic features of ISH *in toto*. Infantile systemic hyalinosis is a condition characterized by widespread deposition of hyaline material in many tissues. Infantile systemic hyalinosis presents hyperpigmented skin over bony prominences with progressive joint contractures, osteopenia, massive gingival hypertrophy, skin abnormalities, severe chronic pain, widespread deposition of hyaline material in many tissues and

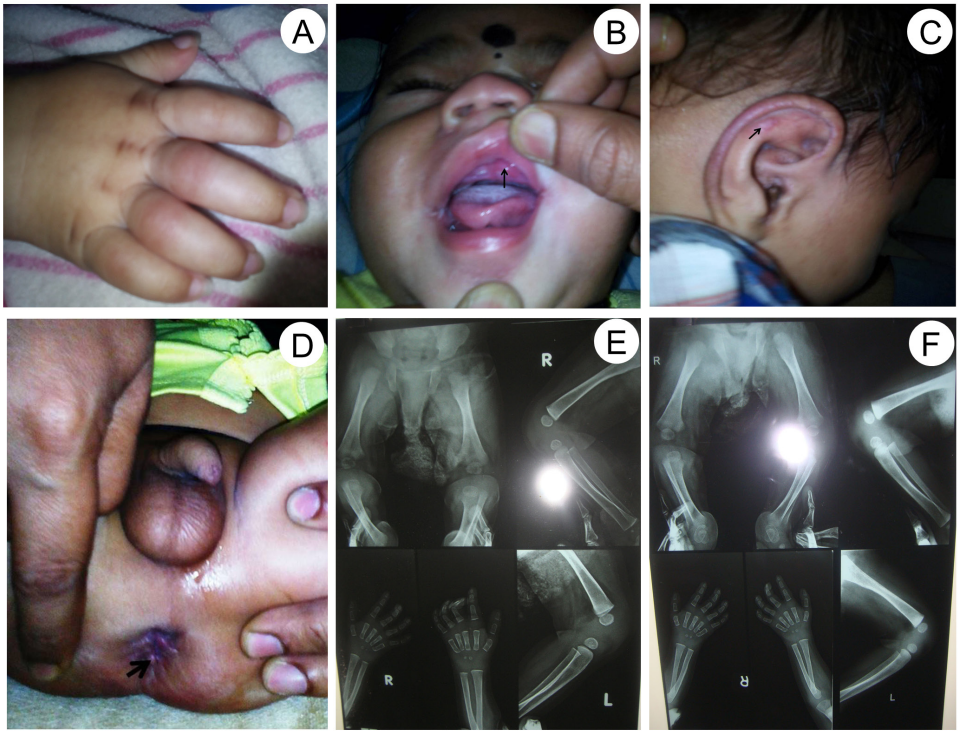

Figure 4 Images of different regions affected in infantile hyalinosis in identical twins. (A) showing Progression of joint contractures in the hand; **(B)** showing Gingival hypertrophy (Arrow mark); **(C)** showing Reddishness and papule over earlobe (Arrow mark); **(D)** showing Papular nodules in the perianal region (Arrow mark); **(E)** & **(F)** radiographs showing the joint contracture.

small pearly papules on face, and perianal regions. Survival beyond 3-4 years of life may become difficult because of impaired chest wall movement, malnutrition, protein losing enteropathy, osteopenia, intractable diarrhea and sepsis involving multi organ systems. Recent investigations documented the use of d-pencillamine has an inhibitory effect on abnormal collagen maturation and shows some improvement in joint mobility (*10*).

The gene responsible for hyalinosis is chromosome 4q21.21 and deletion mutations in the *ANTXR2* gene (anthrax toxin receptor 2 gene) or *CMG2* (capillary morphogenesis protein gene 2) gene cause infantile systemic hyalinosis (*2,3,9*). *TheANTXR2* gene provides instructions for making a protein involved in the formation of tiny blood vessels (capillaries) and is also important for maintaining the structure of basement membranes, which are thin, sheet-like structures that separate and support cells in many tissues. In ISH the defective synthesis of glycosaminoglycans which results in the abnormalities in collagen synthesis and in the *CMG2* gene that binds to type 4 collagen and laminins which provide strength to the basement membrane (*11,12*). The accumulation of an abnormal collagen in different parts of the body is seen in ISH which is responsible for the symptoms. Mutations in the *ANTXR2* gene disrupt the formation of basement membranes, allowing the hyaline substance to leak through and build up in various body tissues (*13*). This condition is inherited in an autosomal recessive pattern, and the parents of an individual with an autosomal recessive condition each carry one copy of the mutated gene, but typically do not show signs and symptoms of the condition.

ISH has to be differentially diagnosed with Farber's disease (*14*), I- cell disease which is a storage disorder (*12*), Stiff skin syndrome (*15*), Winchester syndrome (*16*), Pseudo-Hurler polydystrophy, Lipoid proteinosis and Caffey disease (*17*), and congenital generalized fibromatosis (*18*).

The present case is diagnosed as ISH because it reveals all of the symptoms which are characteristic of ISH and it was ruled out to be any of the above mentioned diseases because of its completion of characteristic symptoms.

ISH can be confirmed by demonstration of hyaline material in the dermis by light microscopy with PAS stain and electron microscopy which reveals cells filled with fine fibrillary material with an enlarged endoplasmic reticulum and golgi apparatus (*19*) also, by intestinal biopsy which reveals villous atrophy, edema, lymphangiectasia, and hyalinosis, and molecular genetic testing for gene *ANTXR2* (*17*). Further, sequencing of the *ANTXR2* gene carried out in blood samples of the present study confirms the clinical diagnosis of infantile hyalinosis. This test has revealed the sample was homozygous for insertion mutation c.277_278insATTATTT (or p.L93Yfs*14) in exon 3, leading to premature termination of protein.

Management of ISH can be done by initial diagnosis,

followed by nutritional, immune and intestinal malabsorption evaluation and echocardiogram evaluation because the heart is involved, to increase life span and prevent recurrence of infections.

To conclude the present case of twins exhibiting infantile systemic hyalinosis is a rare and uncommon genetic disorder of third degree parents confirmed as victims of ISH and not responding well to treatment compared to normal infants. This being the first case of ISH in India planning therapeutic strategies is difficult for pediatricians and other physicians. Hence awareness has to be raised to mutation in the *ANTXR2* gene by explaining the risk of recurrence in future siblings being 25%. Prenatal diagnosis is possible by fetal DNA analysis at around 12 to 16 weeks of pregnancy. Knowledge regarding ISH needs to be updated by clinicians. This is the first case of ISH reported in identical male twins to the best of our knowledge.

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Letter

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Nailfold capillaroscopic changes in Kindler syndrome

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Summary **Kindler syndrome (KS), the fourth major type of hereditary epidermolysis bullosa (HEB), is a rare, autosomal recessive disorder characterized by skin fragility and blistering at birth followed by development of marked photosensitivity and progressive poikilodermatous skin changes in later years. We reported here the case of a 54-year-old woman, who fulfills the diagnostic criteria of KS type of HEB, putting accent on the nailfold capillaroscopic changes. Using videocapillaroscopy we observed pronounced alterations in finger nail capillaries including reduction in capillary density, features of neoangiogenesis (architectural derangement, elongated loops, extremely tortuous, bushy or branching capillaries, thin, branching and interconnected capillaries), enlarged and giant capillaries. We consider the changes observed as an adaptive mechanism that compensate the loss of capillaries due to chronic periungual trauma. Further studies with larger number of patients are needed to confirm the significance of capillaroscopy findings for patients with HEB.**

Keywords: Kindler syndrome, capillaroscopy

Kindler syndrome (KS), the fourth major type of hereditary epidermolysis bullosa (HEB), is a rare, autosomal recessive disorder characterized by skin fragility and blistering at birth followed by development of marked photosensitivity and progressive poikilodermatous skin changes in later years. After the first description by Theresa Kindler in 1954, more than 250 cases have been reported to date (*1-4*). In Bulgaria, about 100 patients suffered from HEB were currently registered. Among them only one case with KS is described (*5*). We report here a new case of KS putting accent on the nailfold capillaroscopic changes.

A 54-year-old woman presented with history of recurrent blistering after minor friction or trauma started after birth. The changes were more prominent on the extremities and tend to regress with age. Subsequently, photosensitivity, discoloration and atrophy of the skin developed. In addition, occasionally gingival and urethral bleeding, and surgically treated squamous cell carcinoma on the dorsum of the right hand 7 years ago were reported. The family history was negative. On examination, diffuse poikiloderma (atrophy, telangiectases, and reticular pigmentation), mainly on the face and dorsal surfaces of the hands and feet, was observed. The dorsum of the hands and feet had atrophic skin with cigarette paper-like wrinkling. There were also skin erosions, atrophic scars, ectropion, gingivitis and periodontitis with missing teeth, and nail changes (prolonged eponychium, transverse and longitudinal ridges, onycholysis, yellow discoloration) (Figure 1). Routine blood tests, including immunological tests, were within normal range. The result from the nerve conduction study indicated the presence of sensory polyneuropathy. The histopathologic examination of skin lesion, performed 20 years ago, revealed an atrophic epidermis, subepidermal cracks, flattened dermis with thin collagen fibers, edema, dilated capillaries, pigmentary incontinence, and scarce perivascular infiltrate. A diagnosis KS type of HEB was made and symptomatic treatment was applied.

In order to explore the changes in microcirculation we performed nailfold videocapillaroscopy at varied magnifications (×60, ×200, and ×500) using digital dermatoscope DinoLite (AnMo, Taipei, Taiwan). The following capillaroscopy findings were found: skin transparency: good; number: 4-6/mm, reduction in capillary density; morphology: shape heterogeneity and marked tortuosity with varied appearance; dimensions: regularly and irregularly enlarged (width 30-50

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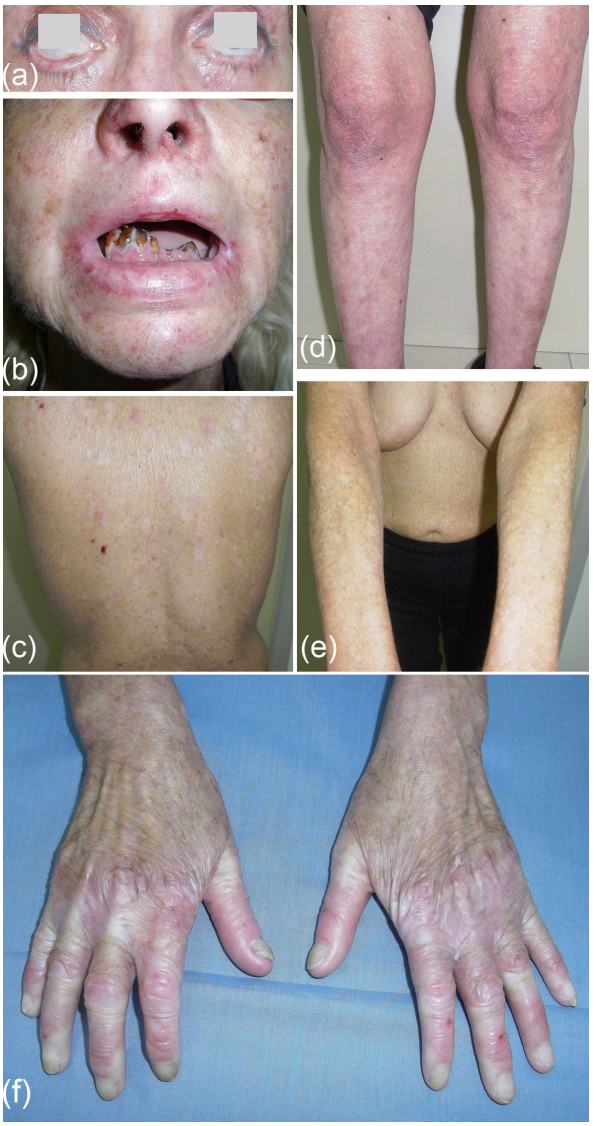

Figure 1. Ectropion (a); periodontitis (b); erythema, atrophy, hyper- and hypopigmented macules, teleangiectasia, xeroderma, atrophic scars (b-e), atrophic scarring with shiny cigarette paper-like wrinkling and small ulcerations on dorsum of the hands (f).

micron) and giant capillaries (width > 50 micron); distribution: architectural derangement and features of neoangiogenesis; blood flow: normal; absence of hemorrhages and thromboses. The interpretation was: presence of nailfold capillary microangiopathy (Figure 2).

KS is caused by a deficiency of the protein kindlin-1 due to a mutation in the gene KIND1 mapped to chromosome 20p12. It manifests during the neonatal period and infancy with skin fragility and trauma-induced skin blistering, which usually resolve with age. Later, varying degrees of photosensitivity and progressive poikiloderma develop. Face, hands and feet are most affected. Ocular and nail changes, periodontitis, esophageal, anal, vaginal and urethral stenosis have been also observed. Additionally, KS patients present an increased susceptibility for development of squamous cell carcinomas. The diagnosis is essentially clinical but could be supported by histopathologic examination, immunostaining with anti-kindlin-1 antibody, electron microscopy examination, and molecular genetic testing (*1-4*).

In 2005, Angelova-Fischer *et al*. (*5*) proposed the following diagnostic criteria for the syndrome: *i*) Major criteria: acral blistering in infancy and childhood, progressive poikiloderma, skin atrophy, abnormal photosensitivity, gingival fragility and/or swelling; *ii*) Minor criteria: syndactyly and mucosal involvement (anal, esophageal, urethral, laryngeal stenosis); *iii*) Associated findings: nail dystrophy, ectropion, palmoplantar keratoderma, leukoplakia, squamous cell carcinomas, skeletal abnormalities, periodontitis and tooth decay. The presence of 4 major criteria makes the diagnosis of KS certain. The presence of 3 major and 2 minor criteria makes the diagnosis probable and the presence of 2 major criteria and 2 minor criteria or associated symptoms renders the diagnosis likely. Our patient had all 5 major criteria, and mucosal and nail involvement, ectropion, squamous cell carcinoma, periodontitis and poorly preserved teeth.

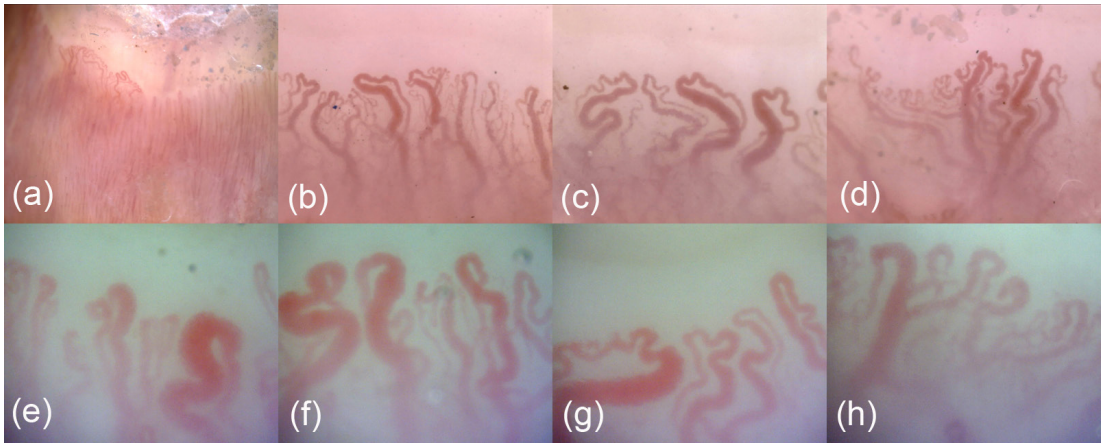

Figure 2. Capillaroscopic findings: reduction in capillary density, shape heterogeneity, marked tortuosity with varied appearance, enlarged and giant capillaries, architectural derangement, neoangiogenesis. Magnification: ×60 **(a)**; ×200 **(b-d)**; ×500 **(e-h)**.

So far there are not studies on microcirculation in HEB and KS in particular. That is why we aimed to evaluate the changes of nail fold microcirculation using non-invasive, digital videocapillaroscopy. We observed pronounced alterations in finger nail capillaries with the main feature of capillary neoformation including elongated loops, extremely tortuous, bushy or branching capillaries, thin, branching and interconnected capillaries. The presence of enlarged and giant capillaries makes the differentiation with Raynaud phenomenon somewhat difficult. Moreover, the clinical appearance of the patient's skin of the hands impress on general practitioners and rheumatologists that she suffer from autoimmune connective tissue disease. However, the patient did not report any vasospastic episodes of her fingers provoked by cold and the repeatedly immunological tests were normal. We consider the neoangiogenesis observed as an adaptive mechanism that compensate the loss of capillaries due to chronic periungual trauma. The role of sensory polyneuropathy could be additionally discussed. However, it is probably a consequence of the general disorder, too.

To the best of our knowledge, this is the first report of microcirculatory alterations in KS. Further studies with larger number of patients are needed to confirm the significance of capillaroscopy findings for patients with HEB.

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Letter

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China takes an active role in combating an Ebola outbreak: On-site observations and reflections from a Chinese healthcare provider

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Summary **As one of the active participants in the global fight against the 2014 outbreak of Ebola virus disease (EVD) in West Africa, China supplied many resources, including medical experts and scientists as well as medical supplies, to the affected countries. A member of the first contingent of Chinese public health experts who worked in Sierra Leone for 65 days, I am pleased to have this opportunity to review the major work done by our team to help deal with the Ebola epidemic in Sierra Leone. This is the first time that a Chinese public health training team has worked in West Africa. The team provides trainings for people from local communities in an effort to encourage local residents to get involved in the war against Ebola. However, the implementation of active measures against Ebola in West Africa was hampered somewhat by certain drawbacks in the area in terms of the health system, the shortage of medical resources, the high illiteracy rate, unhealthy lifestyles, and traditional funeral rites. All of these aspects need to be gradually improved in the aftermath of Ebola, and I believe that this is an area in which the Chinese public health system can play an important role.**

Keywords: Ebola virus disease, Sierra Leone, public health system, China's role

In light of strengthening global ties, an outbreak of Ebola virus disease (EVD) in West Africa in 2014 posed a serious threat to global public health. According to data from the World Health Organization (WHO), there were 28,388 confirmed, probable, and suspected cases of Ebola in Guinea, Liberia, and Sierra Leone prior to September 30, 2015 and 11,296 deaths (*1*). Seventy-five percent of human emerging infectious diseases are transmitted by animals (*2*), and the Ebola virus (EBOV) is thought to be transmitted by fruit bats (*3*). At present, EVD is still believed to be a disease with natural foci, which means that every outbreak originates from the first person who initially becomes infected through contact with bush animals. The virus then spreads rapidly among human beings. Clearly, this epidemic also poses new challenges to the creation of a public health system in China.

The outbreak of EVD severely impacted global public health. During the outbreak, the Chinese Government took quick action by donating money and supplies and by sending a large number of military and civilian medical professionals to the affected countries to combat the epidemic. A member of the first contingent of Chinese public health experts who worked in Sierra Leone for 65 days, I am pleased to have this opportunity to share my frontline experiences and thoughts here.

1. *Major work done by Chinese experts to combat EVD in Sierra Leone*

The major work done by me and other experts can be summarized as follows: *i*) Regular training of Sierra Leone healthcare providers and other professionals in relation to combating EVD. Training courses included information on the epidemiology and transmission of EVD and the current Ebola epidemic, descriptions of Ebola infection and principles of control, and instruction in safety assessment and intervention strategies. *ii*)

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Attending the national Ebola case management meeting organized by the World Health Organization (WHO) and the Ministry of Health of Sierra Leone twice a week to express our views and provide suggestions to help draft guidelines to control Ebola. *iii*) Visiting the Sierra Leone EVD treatment center to gain first-hand practical experience in the treatment and management of EBV and exchanging information on the epidemic with Chinese medical staff and the second contingent of the EBV training team. *iv*) Noting the progress of control of the epidemic and informing experts in China of the most recent guidelines for managing EBV. These efforts helped to establish rules and protocols for fighting the cross-border spread of the disease and also to prepare medical facilities to admit patients with EVD or patients suspected of having EVD.

2. *More active therapy can reduce the mortality rate*

The outbreak of EVD was a terrible disaster, but it was also a rare learning opportunity for medical personnel to learn how to prevent and control epidemics from occurring again (*4*). The main clinical symptoms of EVD are severe gastrointestinal symptoms in the form of vomiting and severe diarrhea, which lead to fluid loss, metabolic abnormalities, and hypovolemic shock. When the patient is unable to drink water, fluids must be administered intravenously. However, a number of critically ill patients died because they failed to receive sufficient fluids. Obviously, if patients receive more active treatment and supportive care, especially with regard to sufficient fluids and preventing and correcting electrolyte abnormalities, then the EVD mortality rate could be drastically reduced (*5*). Although there were reports indicating that some patients in the ICU received excessive fluids that caused pulmonary edema, the delay and lack of intravenous fluids is still a common problem at Ebola treatment centers. According to current statistics, Ebola treatment centers reduced the Ebola mortality rate to 39% merely by providing active rehydration therapy. Some experimental treatments and vaccines are being developed or are currently available. Several patients received a transfusion of serum from previous patients who recovered from EVD so that they can acquire antibodies against EBOV via passive immunization. Passive immunization can also be accomplished by obtaining antibodies against EBOV from infected animals. ZMapp (*6*) is a combination drug that includes many monoclonal antibodies and that has been used to treat patients with EVD, some of whom recovered. Several antivirals, *e.g*. Favipiravir (anti-influenza virus drugs) and Brincidofovir, have demonstrated efficacy when used to treat patients with an early stage of the disease in preliminary clinical trials (*7*). The TKM-Ebola injection, made by the Canadian company Tekmira Pharmaceuticals, blocks the replication and transmission of EBOV. However, there are serious problems with the clinical evaluation of vaccines and their therapeutic efficacy (*8,9*).

3. *The Chinese public health system should play a greater role in the aftermath of Ebola*

Working on the frontlines to prevent and control the epidemic, I realized that the national response center in West Africa plays a leading role in organization, coordination, management, and implementation of a series of rapid responses to control the outbreak. The health minister is in charge of all control efforts. He holds a meeting for relevant personnel to exchange information and he coordinates daily efforts by international organizations, and he then reports directly to the Council of Ministers and the President. Continued and comprehensive guidance, sufficient manpower, and a large amount of supplies provided by the international community greatly helped to effectively combat the epidemic in countries stricken by Ebola.

Multi-sector control efforts used in China played an important role in controlling the epidemic in West Africa. This was the first time a Chinese public health training team has worked in West Africa. The team trains people from local communities in order to encourage local residents to become involved in the war against Ebola. However, the implementation of active measures against Ebola in West Africa was hampered somewhat by drawbacks in the area in terms of the health system, the shortage of medical resources, the high illiteracy rate, unhealthy lifestyles, and traditional funeral rites. All of these aspects need to be gradually improved in the aftermath of EVD, and I believe that this is an area in which the Chinese public health system can play an important role. Thanks to the support of the United Nations Mission for Ebola Emergency Response (UNMEER) and other partners (including the Chinese Government), the three affected countries now have the ability to isolate and treat patients diagnosed with EVD and they have sufficient resources to ensure that the bodies of the deceased are treated in a safe and dignified manner.

The American CDC has many permanent agencies in African countries, and they have played a leading role in the prevention and control of this epidemic. Though various infectious diseases have been effectively controlled in China, the study of tropical diseases and parasitic diseases should not stop in China. Moreover, this study needs to be extended to Africa. Chinese-built hospitals have long played an important role in Africa, so existing health care networks should be used to create a disease prevention and control system. After an outbreak of EVD, the Chinese CDC should send personnel abroad. Given these needs, a specialized agency should be created to implement Chinese multi-sector control efforts in foreign countries. The agency should be organized by the Ministry of Health and

Family Planning Commission and receive a regular budget so that its effectiveness is ensured.

China can organize training courses for public health professionals from African countries and allow trainees to practice at all levels of the disease prevention and control system. A model zone could be initially established and then gradually replicated elsewhere. China assisted in the construction of level 3 biosafety laboratories in Africa. These laboratories were rationally designed and efficiently organized, and they have the ability to effectively protect laboratory personnel and the surrounding environment. The laboratory in Sierra Leone was built with Chinese aid, and it plays an important role in combating Ebola and studying other tropical diseases.

This is the first time that Chinese public health personnel have been sent abroad on such a large scale, and we should compile our experiences and lessons. The dispatching procedures will be optimized and various standard operating procedures (SOPs) will be modified in order to alleviate the concerns of volunteers. Moreover, reasonable standards will be established to ensure logistical support and limit occupational exposure.

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