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Original Article

Semaphorin 3A in Ankylosing Spondylitis

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Biomarker

Abstract *Background/Purpose:* To determine serum semaphorin 3A (Sema 3A) levels in ankylosing spondylitis (AS).
Methods: Serum Sema 3A was measured in 46 AS patients and 30 healthy controls (HCs). For the patients, we recorded demographic data, disease activity, functional index & global assessment, detected human leukocyte antigen-B27 (HLA-B27), and measured erythrocyte sedimentation rate (ESR) & C-reactive protein (CRP).
Results: Sema 3A was higher in AS patients than in HCs (3.98 ± 2.57 *vs.* 1.34 ± 0.48 ng/ml, $p = 0.013$). Area under the curve (AUC) of standard receiver operating characteristic (ROC) has suggested that Sema 3A > 2 ng/ml is better to predict the higher Bath Ankylosing Spondylitis Disease Activity Index (BASDAI, > 4) than ESR or CRP. There were good correlations between higher Sema 3A and uveitis, Schöber's test, as well as interstitial lung disease. AS patients undergoing anti-tumor necrosis factor therapies for 3 months exhibited a positive correlation of change in Sema 3A (ΔSema 3A) with disease activity fluctuation [ΔBASDAI, ΔBath Ankylosing Spondylitis Functional Index (BASFI) and ΔBath Ankylosing Spondylitis - Global score (BAS-G)].
Conclusion: Serum Sema 3A level was increased in AS patients and was inversely correlated to Schöber's test. Serum Sema 3A is better as a bio-marker than ESR or CRP to correlate with high

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disease activity in AS patients, and it is also a good indicator for monitoring disease activity and functional status during anti-TNF treatment. Also, Sema 3A may be taken as a predictor for extra-articular presentations in AS, but this needs further study to elucidate.

Introduction

Ankylosing Spondylitis (AS), a prototype of seronegative spondyloarthritis (SpA), is a chronic auto-inflammatory rheumatic disease with characteristics of sacroiliitis, inflammatory back pain, peripheral arthritis, enthesitis and anterior uveitis.[1,2] Abnormal bone remodeling around enthesis with new bone formation such as syndesmophyte can result in "bamboo spine" with significant limitation of spinal motion in these patients.[3,4] Eventually, the disease can lead to heavy economical and mental burdens in patients, their families and the whole society. Therefore, it is important to find out a potential bio-marker for monitoring disease activity and developing better approaches for the prevention and treatment of this kind of irreversible skeletal disease.

Notably, increasing evidence has suggested that bone remodeling is regulated by a variety of factors.[5,6] Semaphorins are a large family of secreted and membrane-bound proteins originally discovered in the nervous system, which are implicated in repulsive axon guidance during nerve development.[7] Semaphorin 3A (Sema 3A) is a prototype of axonal guidance molecule in semaphorin family. For example, Sema 3A regulates the timing of tooth innervations as well as dental axon navigation and patterning.[8,9] Previous studies have also demonstrated the implication of Sema 3A in skeletal system.[10,11] All three bone cell lineages (osteoclast [OC], osteoblast [OB] and osteocyte) express Sema 3A and its receptors.[12] Sema 3A could inhibit the migration of OC precursor cells by suppressing *RhoA* activation. On the other hand, OB differentiation is stimulated and adipogenesis is suppressed by Sema 3A through the activation of canonical Wingless (WNT)/β-catenin pathway.[13]

Other studies have pointed to the involvement of semaphorins in the regulation of immune system. Thus, it is also denoted as "immune semaphorins".[14,15] Sema 3A is recognized as a potent regulator during immune responses, from the early initiation to the late subsiding phase of inflammatory reaction.[16] Sema 3A expression has been found increased in differentiating macrophages and activated T cells, suggesting that it has a role in modulating inflammatory conditions.[17] The altered expression of Sema 3A in T cells has been shown relevant to the progression of rheumatoid arthritis (RA),[18] and low serum Sema 3A levels in systemic lupus erythematosus (SLE) patients have also been found correlated to SLE Disease Activity Index (SLEDAI) scores, reflecting disease activity.[19]

In the present investigation, we measured serum Sema 3A in AS patients and health controls (HCs) and tried to find its relevance to the imbalance between bone formation and resorption in AS. In addition, we also aimed to figure out correlations between Sema 3A level and inflammatory reactants, disease activity, physical mobility and extra-articular manifestation in this particularly auto-inflammatory disease with predominantly excessive enthesopathic new bone formation. Besides, we recorded the changes in Sema 3A in some AS patients who received anti-TNF therapies for 3 months.

Materials and methods

Patients and controls

The study was approved by the Institutional Review Board of Taipei Veterans General Hospital. Informed consents were obtained from all participants, and the results were analyzed. We consecutively enrolled 46 Taiwanese AS patients (36 men and 10 women) from the Outpatient Department, who were fulfilling the 1984 modified New York criteria for diagnosis of AS[20] without the previous treatment of systemic steroid. Blood samples were obtained from all patients and 30 age- and sex-matched HCs. These HCs were healthy voluntary blood donors without any occult rheumatic diseases. Clinical and laboratory assessments were performed on the blood sampling day. We evaluated disease activity in AS patients by the Bath Ankylosing spondylitis Disease Activity Index (BASDAI), functional ability by the Bath Ankylosing spondylitis Functional Index (BASFI) and global assessment by the Bath Ankylosing Spondylitis - Patient Global Score (BAS-G).[21–23] We also recorded all AS patients' gender, disease duration, axial involvement and/or peripheral arthritis, uveitis, lung involvement (i.e., interstitial lung disease [ILD] as shown in a chest X-ray which was assessed by radiologists), Schöber's test, finger to floor distance, chest expansion, right/left lateral bending, occipital to wall, tragus to wall, intramalleolar distance and degrees of the right/left lateral rotation of cervical spine. There were neither known history of osteoporosis nor bony fractures among all subjects. Of all AS patients, 4 had undergone etanercept & another 4 had undergone adalimumab therapies for 3 months (under national reimbursement program).

Serum level of Sema 3A

Serum samples were collected from all subjects as they entered the study. Samples of peripheral venous blood were allowed to clot and centrifuged at 2000 rpm for 15 min to obtain sera, which were then snap-frozen at −80 °C and kept until use. A commercial ELISA kit (Cusabio Biotech Co.,

Baltimore, Maryland, USA) was used to measure serum Sema 3A. The detection range of ELISA kits for serum Sema 3A is 0.156—10 ng/ml according to the manufacturers.

Measurement of ESR, CRP and HLA-B27

ESR was measured by Westergren method, CRP by nephelometry (Beckman IMMAGE 800; Beckman, Fullerton, California, USA) and HLA-B27 typing by flow-cytometry (Becton-Dickinson Pharmingen, San Diego, CA, USA).

Statistical analysis

Statistical calculations were carried out using the SPSS for Windows (version 17) (SPSS, Chicago, Illinois, USA). Data were represented as the mean ± standard deviation for continuous variables and as proportions for categorical variables. *P* values were provisionally regarded as significant if they were less than 0.05. The Mann—Whitney *U* test, Fisher's exact test, Spearman's rank correlation and point-biserial correlation were used to analyze group differences and associations. Receiver operating characteristic (ROC) curve plot analysis with its area under the curve (AUC) was used to evaluate and compare the goodness of each biomarker.

Results

Demographic data

The clinical characteristics of 46 AS patients were shown in Table 1. The male to female ratio was 3.6:1 (78.3%: 21.7%). The age and disease duration of these patients were 38.4 ± 12.4 and 13.7 ± 10.1 yrs, respectively. There was neither sex nor age difference between AS patients and HCs. Among these AS patients, 30 had both axial and peripheral joint involvement while 16 suffered from axial disease only. Nine patients had uveitis. Five were diagnosed with ILD by radiologists via chest X-ray. HLA-B27 was positive in 41 (89.1%) patients. ESR and CRP levels were 38.7 ± 12.6 mm/h and 3.36 ± 1.74 mg/dl, respectively. Besides, BASDAI, BASFI, BAS-G, Schöber's test, finger to floor, chest expansion, right lateral bending, left lateral bending, occipital to wall, tragus to wall, and intramalleolar distance, degrees of the right/left cervical spine lateral rotation were also recorded.

The serum levels of Sema 3A in AS patients and HCs

We measured serum levels of Sema 3A in all AS patients and HCs (Table 2). A comparison showed that Sema 3A was

Table 1 Demographic and clinical characteristics of the 46 AS patients and 30 healthy controls (HCs).

Characteristic	AS (n = 46)	HCs (n = 30)	*p*-value
Age (yrs old)	38.43 ± 12.36	34.62 ± 10.16	NS
Male/female	36/10	24/6	NS
Disease duration (yr)	13.7 ± 10.1		
Axial with peripheral arthritis/axial disease only (patient number)	30/16		
Uveitis/no uveitis	9/37		
Interstitial lung disease (by CXR)	5		
HLA-B27 (+) (%)	41 (89.1%)		
CRP (mg/dl)	3.36 ± 1.74		
ESR (mm/h)	38.7 ± 12.6		
BASDAI	4.8 ± 2.2		
BASFI	3.2 ± 2.3		
BAS-G	5.3 ± 2.8		
Schöber's test (cm)	3.07 ± 1.77		
Finger to floor (cm)	18.76 ± 16.36		
Chest expansion (cm)	4.05 ± 1.96		
Right lateral bending (cm)	9.14 ± 6.08		
Left lateral bending (cm)	9.28 ± 6.83		
Occipital to wall (cm)	4.16 ± 7.49		
Tragus to wall (cm)	13.49 ± 5.61		
Intramalleolar distance (cm)	95.43 ± 24.61		
Cervical spine lateral rotation, right (degree)	46.4 ± 31.7		
Cervical spine lateral rotation, left (degree)	45.4 ± 29.5		

Abbreviations: AS: Ankylosing spondylitis; HC: healthy control; NS: non-significant; HLA-B27: human leukocyte antigen-B27; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; BASDAI: Bath Ankylosing spondylitis Disease Activity Index; BASFI: Bath Ankylosing spondylitis Functional Index; BAS-G: Bath Ankylosing spondylitis - Patient Global Score; CXR: chest X ray; yr: year. Data are presented as mean ± standard deviation.

Table 2 Serum levels (ng/ml) of Sema 3A in 46 AS patients and 30 healthy controls (HCs).

	AS (46)	HCs (30)	*p*-value
Sema 3A (ng/ml)	3.98 ± 2.57	1.34 ± 0.48	0.013†

Abbreviations: AS: Ankylosing spondylitis; HC: healthy control; Sema 3A: semaphorin 3A. Data are presented as mean ± standard deviation. †$p < 0.05$: significant difference.

Table 3 Correlations of Sema 3A to Schöber's test, uveitis or interstitial lung disease in 46 AS patients.

	Schöber's test	Uveitis	Interstitial lung disease (by CXR)
Sema 3A (ρ/p)	−0.492/0.047*	0.434/0.072	0.474/0.053

Abbreviations: AS: Ankylosing spondylitis; CXR: chest X-ray; ρ: correlation coefficient. $p < 0.05$ and *: significant difference.

significantly higher in AS patients than in HCs (3.98 ± 2.57 *vs.* 1.34 ± 0.48 ng/ml, $p = 0.013$).

Correlation of Sema 3A levels to the demographic data in AS patients

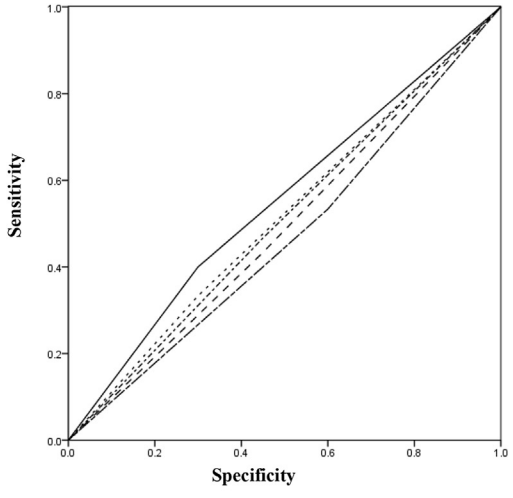
Comparisons between different parameters in 46 AS patients were made. Table 3 shows negative correlation between serum Sema 3A and Schöber's test ($\rho = -0.492$, $p = 0.047$). On the other hand, Sema 3A tended to positively correlate to ILD and uveitis ($\rho = 0.474/p = 0.053$ and $\rho = 0.434/p = 0.072$, respectively). There were no significant relationships between Sema 3A levels and BASDAI/BASFI/BAS-G (data not shown).

ROC curve analysis to evaluate the potential usefulness of Sema 3A, ESR and CRP as predictors for AS patients with high disease activity

To evaluate whether different serum levels of Sema 3A, ESR and CRP could be used as indicators for monitoring high disease activity in AS (BASDAI ≥ 4),[24] the ROC curve with its AUC for each parameter was calculated (Fig. 1). The AUC positively correlated to BASDAI (>4) included Sema 3A > 2 ng/ml, Sema 3A > 3 ng/ml, ESR> 25 mm/h, ESR> 28 mm/h and CRP> 1.0 mg/dl in a descending order (with AUC of 0.550, 0.517, 0.508, 0.492 and 0.467, respectively).

The predicative value of Sema 3A for various clinical manifestations in AS patients

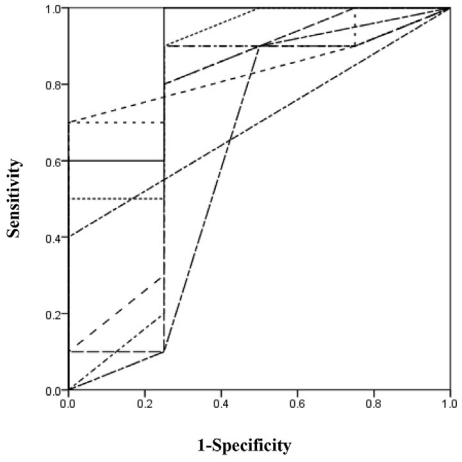
We calculated the AUCs with Sema 3A larger than 2 ng/ml versus different clinical manifestations in AS (Fig. 2). The largest AUC was 0.900, in case of lateral bending distance *vs.* Sema 3A (>2 ng/ml), followed by 0.863 (Schöber's test *vs.* Sema 3A > 2 ng/ml), 0.838 (chest expansion *vs.* Sema 3A > 2 ng/ml), 0.738 (cervical spine lateral rotation *vs.* Sema 3A > 2 ng/ml), 0.725 (intra-malleolar distance *vs.* Sema 3A > 2 ng/ml), 0.700 (peripheral arthritis *vs.* Sema 3A > 2 ng/ml) and 0.613 (uveitis *vs.* Sema 3A > 2 ng/ml) in a descending order.



Test Result Variable(s)	Area Under the Curve
Sema 3A > 2 ng/mL	0.550
Sema 3A > 3 ng/mL	0.517
ESR > 25 mm/hr	0.508
ESR > 28 mm/hr	0.492
CRP ≥ 1.0 mg/dL	0.467

ROC curves for high BASDAI (>4)

Figure 1. ROC curve of high BASDAI (≥4) in AS patients. BASDAI ≥4 indicates presence of high disease activity. In ROC curve, the larger the AUC of BASDAI is, the more the predictive value exists. The best predictor for higher BASDAI is the Sema 3A > 2 ng/ml.



Diagonal segments are produced by ties

Source of the Curve

- Left lateral bending distance
- Right lateral bending distance
- Schöber's test
- Chest expansion distance
- Right cervical spine lateral rotation
- Left cervical spine lateral rotation
- Intra-malleolar distance
- Peripheral arthritis
- Uveitis

Test Result Variable(s)	Area Under the Curve
Lateral/ right bending distance	0.900/ 0.863
Schöber's test distance	0.863
Chest expansion distance	0.838
Right/ left Cervical spine lateral rotation	0.738/ 0.713
Intra-malleolar distance	0.725
Peripheral arthritis	0.700
Uveitis	0.613

ROC curves for serum Sema 3A > 2 ng/ml

Figure 2. ROC curve of serum Sema 3A $>$ 2 ng/ml in AS patients. In ROC curve, the larger the AUC is, the more the predictive power for the clinical characteristics of AS exists.

Table 4 Correlations of serum level of ΔSema 3A to ΔBASDAI, ΔBASFI, or ΔBAS-G in 8 AS patients who underwent biological therapy with anti-tumor necrosis factor biologics (4 etanercept and 4 adalimumab) for 3 months.

ΔSema 3A *versus*	ΔBASDAI	ΔBASFI	ΔBAS-G
1st month − baseline	$\rho = 0.314$	$\rho = 0.371$	$\rho = 0.142$
	$p = 0.034^{\dagger}$	$p = 0.043^{\dagger}$	$p = 0.012^{\dagger}$
3nd month − baseline	$\rho = 0.486$	$\rho = 0.657$	$\rho = 0.314$
	$p = 0.017^{\dagger}$	$p = 0.007^{\dagger}$	$p = 0.009^{\dagger}$

Abbreviations: AS: Ankylosing spondylitis; Sema 3A: semaphorin 3A; ρ: correlation coefficient and $^{\dagger}p < 0.05$: significant difference. Δ: represents data at 1 month or at 3 months subtracted from data at baseline; BASDAI: Bath Ankylosing spondylitis Disease Activity Index; BASFI: Bath Ankylosing spondylitis Functional Index; BAS-G: Bath Ankylosing spondylitis - Patient Global Score.

Magnitude of improvement in BASDAI, BASFI & BAS-G as related to concentration change in Sema 3A in AS patients undergoing biologics for 3 months

Eight AS patients underwent anti-TNF therapy (4 etanercept & 4 adalimumab). We measured the changes in indices of Sema 3A, BASFI, BASDAI, & BAS-G before and after biological therapy and calculated the changes in these indices as ΔSema 3A, ΔBASDAI, ΔBASFI and ΔBAS-G. Table 4 shows significantly positive correlations of ΔSema 3A to ΔBASDAI/ΔBASFI and ΔBAS-G at the end of 1st month and 3rd months of treatment ($\rho = 0.314/p = 0.034$, $\rho = 0.371/p = 0.043$ and $\rho = 0.142/p = 0.012$ at 1 month; $\rho = 0.486/p = 0.017$, $\rho = 0.657/p = 0.007$ and $\rho = 0.314/p = 0.009$ at 3 month, respectively). There were higher correlation coefficients (ρ) and lower p values for follow-up after 3 months than after one month.

Discussion

It has been widely accepted that excessive OB activity causing bamboo spine or joint ankylosis is a severe burden to AS patients, with the clinical features of spinal immobility and functional limitation. Indeed, inflammation may induce pain on motion and stiffness on rest, which further result in limited chest wall expansion on inspiration. On the other hand, the associated neuropathy or secondary fibromyalgia might further influence the distance measurement of Schöber's test. In the present investigation, we found serum levels of Sema 3A were increased significantly in AS patients than in HCs and correlated to the shortening of Schöber's distance, although similar findings were not noted in Perrotta's report.[25] Indeed, in the present study, we enrolled more HCs and AS patients than they did. This might more obviously reflect the real world scenario. Besides, AS patients who received systemic steroid treatment were excluded in our study since systemic steroid could significantly interfere with these osteoimmunologic markers. Thus, our results have suggested that Sema 3A might affect range of motion in the spine of AS patients. Although there lacks direct evidence, it is possible that Sema 3A acts as an osteoblastic molecule on the formation of enthesitic new bone formation since it is a WNT/β-catenin stimulating factor as well as an inhibitor on OC precursor cell migration.[13] Previous evidence has suggested

that both central and peripheral nervous systems regulate bone remodeling.[26] Sema 3A is a well-known axon guidance molecule which functions as a key-modulator in the development of the nervous systems.[27,28] Sema 3A exhibits temporal and spatial expression patterns in parallel with the establishment of innervation.[27] Therefore, it is possible that Sema 3A can also modulate bone remodeling indirectly via regulating nervous systems. In AS patients with enthesopathy, syndesmophyte formation and/or inflammatory backache with shortening of Schöber's distance, Sema 3A might provide a link between high bone remodeling with subsequent enthesitic new bone formation and excessive sensory innervations, which subsequently result in pain.

As has been demonstrated in the previous studies, Sema 3A can also act as a potent immune regulator,[14–16] holding back inflammatory responses,[29,30] and blocking collagen-specific proinflammatory cytokine release (such as IFN-γ and IL-17).[31] In this study, we have demonstrated significantly positive correlations of ΔSema 3A to ΔBASDAI, ΔBASFI as well as ΔBAS-G in AS patients who received anti-TNF biologics for 3 months. Besides, an obvious tendency for positive correlation between Sema 3A and uveitis was also seen. These results have suggested that circulating Sema 3A level is high during systemic inflammation in AS and allied diseases and *vice versa*, which is compatible with those reported by Perrotta et al. regarding AS and Costa et al. regarding multiple sclerosis.[25,32] Similar results have also been demonstrated in our previous study on the regulatory T cells in AS patients after anti-TNF therapy.[33] In Burder's and Rimar's studies, Sema 3A and its receptor (neuropilin-1) were expressed in regulatory T cells.[34,35] These imply that in AS patients, enhanced immunoregulatory ability of Sema 3A emerges during inflammatory process, thus triggering a positive feedback to amplify Sema 3A *pe se* and the subsequent inflammation as well as new bone formation or enthesitic syndesmophytes. Further functional study of Sema 3A in AS patients is necessary to elucidate the role of Sema 3A in the pathogenesis of AS and allied diseases.

There is marginally significant positive correlation between Sema 3A and ILD (Table 3, $p = 0.053$). As has been shown in many previous investigations,[34–41] semaphorin members and their receptors can enhance tissue fibrosis including liver cirrhosis, diabetes induced glomerulosclerosis, neural scar tissue formation and rotator cuff injury originated from tendon fibrosis. Thus, it is speculative that Sema 3A might induce similar fibrotic reaction such as pulmonary fibrosis or ILD in AS. On the other hand, inflammatory reactions also contribute to high Sema 3A concentration, which is then perpetuating each other.

Up to now, there still lacks satisfactory serum biomarker for monitoring disease activity and functional status in AS patients that is widely accepted except CRP and ESR. In the present investigation, we used ROC curve and its AUC to assess the goodness of serum Sema 3A, ESR and CRP as a biomarker to reflect the disease activity of AS patients with active inflammation (BASDAI > 4). We found that in case that its level is higher than 2 ng/ml, Sema 3A can be taken as a better surrogate predictor than ESR or CRP for monitoring the disease activity and magnitude of inflammation in AS patients. Moreover, as shown in Fig. 2, when serum Sema 3A is higher than 2 ng/ml, efforts might be put to find overlooked limitations of either axial or peripheral joints movement (such as lateral bending distance, Schöber's test, chest expansion, cervical spine lateral rotation as well as intra-malleolar distance) and extra-articular manifestation (such as uveitis) in AS patients.

There are some limitations in this study. Firstly, we did not measure other bone formation markers such as sclerostin or osteocalcin in AS patients simultaneously, which we plan to check as a reference indices for bone formation at cellular level in the future. Secondly, small number of AS patients and HCs might lead to equivocal conclusions. Thirdly, we did not measure bone mineral density in AS patients because they were predominantly young man without history of bone fracture, who were reluctant to take this examination.

In conclusion, high serum Sema 3A in AS patients demonstrated in the present study has suggested an important role of Sema 3A in the pathogenesis of abnormal bone remodeling, higher activity of sensory nerve innervation to lower pain threshold and immune modulation in AS. Whether serum level of Sema 3A can be taken as a biomarker for monitoring inflammatory activity or as a prognostic predictor for extra-articular involvement in AS patient remains an open question.

Conflicts of interest

All authors declare no conflicts of interest.

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