

Adhesion Molecule CD146 and its Soluble Form Correlate Well with Carotid Atherosclerosis and Plaque Instability

Yi-Ning Qian,¹ Yong-Ting Luo,² Hong-Xia Duan,² Li-Qun Feng,¹ Qi Bi,¹ Yong-Jun Wang³ & Xi-Yun Yan²

¹ Department of Neurology, Beijing Anzhen Hospital, Capital Medical University, Beijing, China

² Key Laboratory of Protein and Peptide Pharmaceutical, CAS-University of Tokyo Joint Laboratory of Structural Virology and Immunology, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China

³ Department of Neurology, Beijing Tiantan Hospital, Capital Medical University, Beijing, China

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Correspondence

X.-Y. Yan, M.D., Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Beijing 100101, China.

Tel.: +86 10 6488 8583;

Fax: +86 10 6488 8584;

E-mail: yanxy@ibp.ac.cn

and

Y.-J. Wang, M.D., Department of Neurology, Beijing Tiantan Hospital, Capital Medical University, No.6 Tiantanxili, Dongcheng District, Beijing 100050, China.

Tel.: 86-010-67098350;

Fax: 86-010-67013383;

E-mail: yongjunwang1962@gmail.com

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SUMMARY

Aims: Intraplaque neovascularization and foam cell infiltration contribute to the development of unstable plaque, leading to thromboembolism and stroke. Cell adhesion molecules (CAMs) have been reported to be involved in the progression of atherosclerosis and plaque vulnerability. The aim of this study was to assess the association of adhesion molecule CD146 with carotid plaque instability. **Methods:** We collected forty atherosclerotic plaques from 40 patients undergoing carotid endarterectomy. The clinical information of each patient was obtained, and the plaque morphology and characteristics were examined by the ultrasound. The CD146 expressions of the plaques were graded by using semiquantitative scales. The serum level of soluble form of CD146 was detected by enzyme-linked immunosorbent assay (ELISA). **Results:** CD146 expression was mainly on the intraplaque blood vessels and infiltrated macrophages. The CD146 expression was strongly correlated with the matrix metalloproteinase-9(MMP-9)expressions ($P < 0.001$) in the plaques. Soluble CD146 (sCD146) was also elevated in patients with atherosclerotic plaques. There was significant correlation between the increased CD146 expression and sCD146 level ($P = 0.0057$). sCD146 correlated well with serum MMP-9 ($P < 0.0044$), IL-6 ($P = 0.0044$) and high sensitivity C-reactive protein (hsCRP) ($P = 0.005$). **Conclusions:** Adhesion molecules CD146 and its soluble form strongly correlated with the development of inflammation of atherosclerosis and plaque instability. CD146 may be a promising biomarker for monitoring the development and instability of atherosclerotic plaque in patients with carotid diseases.

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The first two authors contributed equally to this work.

Introduction

Atherosclerosis (AS) mainly occurred on the large arteries and is characterized by the chronic inflammation in the lesions [1–3]. Changes within the vessel wall of the internal carotid artery (ICA) can lead to plaque vulnerability, which is the main reason for carotid-related cerebrovascular ischemic events [4–7]. It has been reported that inflammatory factors, protease and so on play an important role on the process of plaque vulnerability, such as the matrix metalloproteinases (MMPs), especially the matrix metalloproteinase-9(MMP-9) [8]. The increased level of serum MMP-9 was detected in the atherosclerotic patients with instable plaques compared with those with stable plaques, suggesting the

correlation of MMP-9 and the instability of plaques. The adhesion molecules are also reported to account for the process of plaque vulnerability. The initiation of AS is triggered by endothelial dysfunction and induction of inflammation, accompanied by the increased expression of adhesion molecules that stimulates the adhesion and transmigration of leukocyte into the vascular sub-endothelial space [9,10]. The increased expression of cell adhesion molecules(CAMs) on the vascular endothelial cells, such as intercellular adhesion molecule-1(ICAM-1), vascular cell adhesion molecule-1(VCAM-1), P-selectin, etc., has been reported to play a critical role on the pathogenesis of atherogenesis [11–13]. Monitoring changes of CAMs at the inflammatory sites can help predicting major vascular events.

Although it is difficult to quantify the expression of these molecules on the cell surface *in vivo*, their soluble forms can be detected in serum by specific antibodies using ELISA. Circulating soluble CAMs are the results of cleavages of membrane-bound CAMs. Their concentration in serum/plasma has been associated with the state of cardiovascular disease [14,15]. Detecting the serum soluble CAMs may provide evidence for early intervention and secondary prevention of stroke related to carotid diseases, and monitoring effectiveness of treatment.

CD146, an adhesion molecule that was originally identified as a melanoma marker [16], has been studied as angiogenesis marker and reported to be able to upregulate neovascular endothelial cells [17,18], and mediate many chronic inflammatory processes [19–21]. Its soluble form, sCD146, originated from the membrane-bound CD146, has also been well studied in many inflammatory diseases [22–25]. As a chronic inflammatory disease, AS is characterized by neovascularization and inflammatory cell infiltration in lesion plaques. However, there has still little known about CD146 and its sCD146 in AS. We hypothesize that CD146 and sCD146 may also be related with the progression of atherosclerotic lesions.

Patients and Methods

Patients

This study was approved by the Ethics Committee at Beijing Anzhen Hospital, Capital Medical University and the written informed consent was obtained from each patients. Between March 2011 and September 2012, 40 patients (26 men, 14 women; mean age, 65.6 ± 8.25 years) from Beijing Anzhen Hospital with high-grade ($\geq 70\%$) extracranial carotid artery stenosis were selected for carotid endarterectomy. Forty age-matched and sex-matched healthy individuals were selected as controls. Detailed information on the patient and control groups was shown in Table 1. The subjects had any of the following conditions were excluded: acute stroke within 3 months, liver and kidney dysfunction, tumors, systematic inflammatory diseases and autoimmune diseases. Risk factors for atherosclerotic disease, including history of diabetes metabolism, hypercholesterolemia, and levels of high sensitivity C-reactive protein (hsCRP) were recorded in all patients.

The Ultrasound Examination of Carotid Plaques

We performed the carotid ultrasound (US) examinations in the following predefined areas: bilateral distal, bulb, and proximal ICAs. We defined the atherosclerotic plaques as a local thickening of the intima of ≥ 1.5 mm. To evaluate the vulnerability of carotid plaque, we performed high resolution B-mode US, using 7.5 MHz probe (Vivid 7, GE-Vingmed, Horten, Norway). Two independent observers (WJ and ZQ), who were blinded to all of the clinical information and results, rated plaque morphology according to the standard protocol. The characteristics of the plaques were described according to the modified Gray Weale classification [26]. Patients with uniformly hyperechoic or predominantly ($>50\%$) hyperechoic were classified into the stable plaque group. Patients with uniformly hypoechoic or predomi-

Table 1 Clinical information of controls and patients with AS

	Control	AS with stable plaque	AS with vulnerable plaque
No. of patients	40	16	24
Sex (F:M)	16:24	9:7	5:19
Mean age (years)	63.1 ± 10.8	66.3 ± 7.8	64.7 ± 8.2
History of hypertension n (%)	0	14 (87.5%)	19 (79%)
History of DM n (%)	0	2 (12.5%)	10 (41.6%)
History of coronary artery disease n (%)	0	8 (50%)	7 (29.1%)
History of stroke n (%)	0	4 (25%)	12 (50%)
TG (mm)	1.56 ± 1.11	1.70 ± 0.99	1.58 ± 1.31
TC (mm)	4.12 ± 1.09	4.68 ± 0.77	3.73 ± 0.95
LDL-C (mm)	2.23 ± 0.86	2.83 ± 0.88	2.36 ± 0.67
HDL-C (mm)	1.05 ± 0.25	1.11 ± 0.25	0.97 ± 0.25
hsCRP (mg/L)	0.58 ± 0.82	2.22 ± 1.86	3.82 ± 4.89

DM, diabetes metabolism; TG, triglyceride; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; hsCRP, high sensitivity C-reactive protein.

nantly ($>50\%$) hypoechoic were classified into the vulnerable plaque group.

Blood Samples

Fasting blood samples from an antecubital vein were collected and placed at 4°C for half an hour, then centrifuged at 2000 rpm for 5 min. To avoid the loss of biological activity, serum samples were collected as soon as possible, stored at -70°C and thawed just before testing. The total cholesterol (TC) and triglyceride (TG) concentrations in the serum were assayed by routine enzymatic methods. The concentration of low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) were determined by a direct homogeneous assay (Au5421, BECKMAN, COULTER, America). The serum hsCRP level was measured with immunoturbidimetric assay (Diagnostica kit, DiaSys Diagnostic Systems GmbH, Holzheim, Germany).

Tissue Sampling

Forty entire carotid plaques of the human carotid AS lesions were obtained from carotid endarterectomy, then were fixed in neutral buffered formalin and embedded in paraffin. Normal carotid arterial specimens ($n = 5$) served as the control were from the donor of heart transplant operations, fixed in neutral buffered formalin, and then embedded in paraffin.

Antibodies and Reagents

The following proteins and antibodies were used in this study: recombinant human soluble CD146 (Sinobiological Co., Ltd.

Beijing, China); mouse originated anti-CD146 monoclonal antibodies, such as AA1, AA4, and AA98 (generated in Yan's laboratory [18,27]). We used AA1 and AA98 for ELISA [28], and AA4 for immunohistochemistry (paraffin-embedded). We labeled AA98 with biotin in Tianjin Sungene Biotech Co., Ltd.

The following antibodies were also used in this study: anti-human CD31, antihuman CD68, antihuman α -SMA (Abcam); and horseradish peroxidase-conjugated antimouse and antirabbit secondary antibodies (GE Healthcare, Uppsala, Sweden). The serum cytokine, such as IL-6, from patients with AS was detected by the BD Cytometric Bead Array (CBA) human Th1/Th2/Th17 cytokine kit.

Immunohistochemical Staining

For immunohistochemistry staining, deparaffinized paraffin-embedded tissue sections were incubated first with CD146 antibody (AA4), and then with biotin-conjugated antimouse antibody (1:1000), followed by HRP-conjugated streptavidin (Dianova, Rodeo, CA, USA). A ready-to-use solution of 3,3'-Diaminobenzidine was used to observe the expression of CD146. The sections were finally counterstained with hematoxylin. Sections without primary antibody were served as negative controls. For immunofluorescence, deparaffinized sections were stained with antibodies specific for α -SMA, CD68, CD31 or CD146 (AA4) followed by fluorescence-labeled secondary antibodies. The nuclei were counter-

stained with 4', 6-diamidino-2-phenylindole (DAPI). The sections were visualized using a confocal laser-scanning microscope (Olympus).

Detection of sCD146 and MMP-9 by ELISA

Detection of serum sCD146 was performed as described previously [23]. In brief, the serum sCD146 level was determined by ELISA using the capture antibody, AA1 (2 μ g/mL) and the detection antibody, biotin-conjugated AA98 (1.5 μ g/mL); the detection enzyme was HRP-conjugated streptavidin (Dianova). The standard curve was determined using the recombinant sCD146 in phosphate-buffered saline (PBS) buffer (from 80 to 1.25 ng/mL). Serum samples from patients and controls were diluted 1:9 in PBS before measurement. A commercialized solution of 3, 30, 5, 50-tetramethylbenzidine (TMB) was used as a HRP enzyme substrate. A wavelength of 450 nm sample absorption was measured using a BioRad ELISA reader (Richmond, CA, USA). The level of MMP-9 in serum samples was determined using commercialized enzyme-linked immunosorbent assay kit according to manufacturer's instructions.

Statistical Analysis

SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) software was used to perform the statistical analyses. All the measurement data was

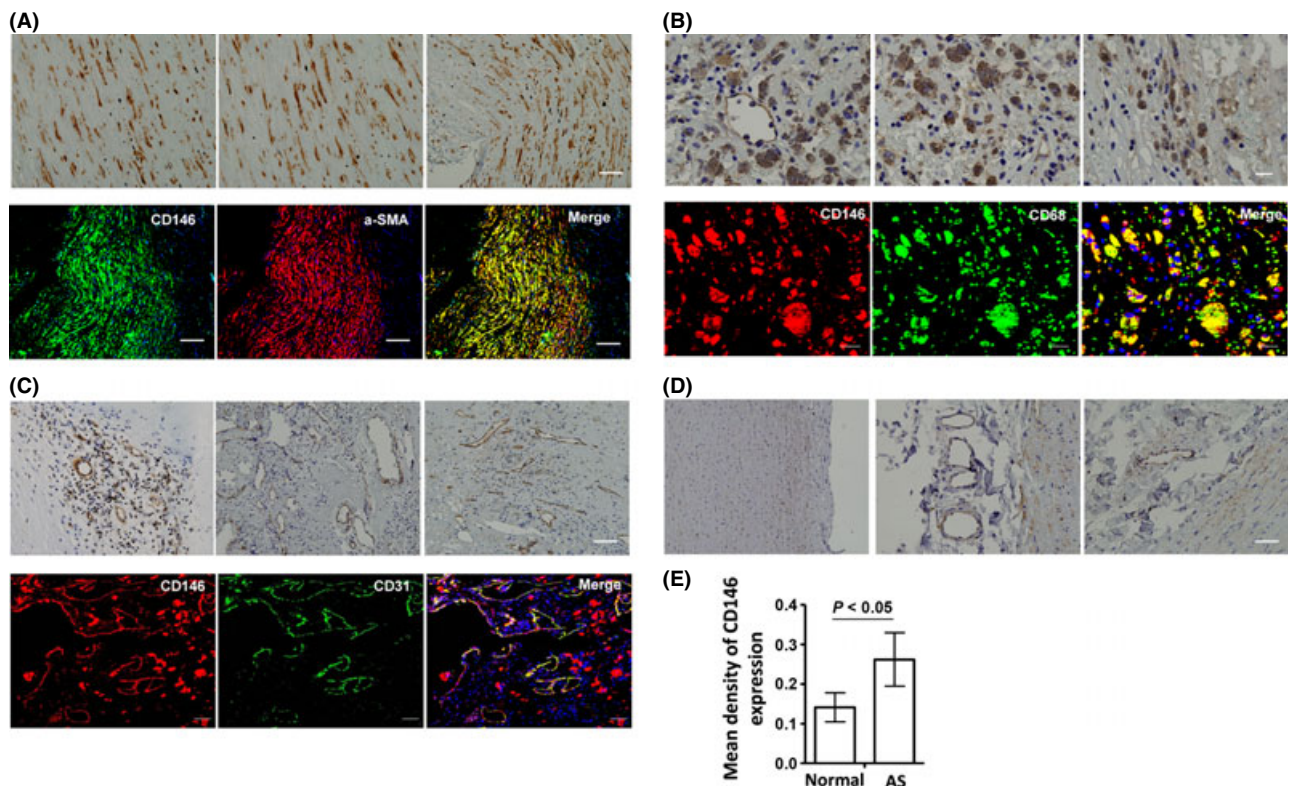


Figure 1 CD146 expression on AS plaques and normal artery. (A) CD146 expression on the smooth muscle cells in the plaques. (B) CD146 expression on the macrophages in the plaques. (C) CD146 expression on the endothelial cells in the plaques. (D) CD146 expression in the normal artery. (E) The expression of CD146 is increased in the blood endothelial cells of plaques ($n = 40$) compared with that in the normal artery ($n = 5$).

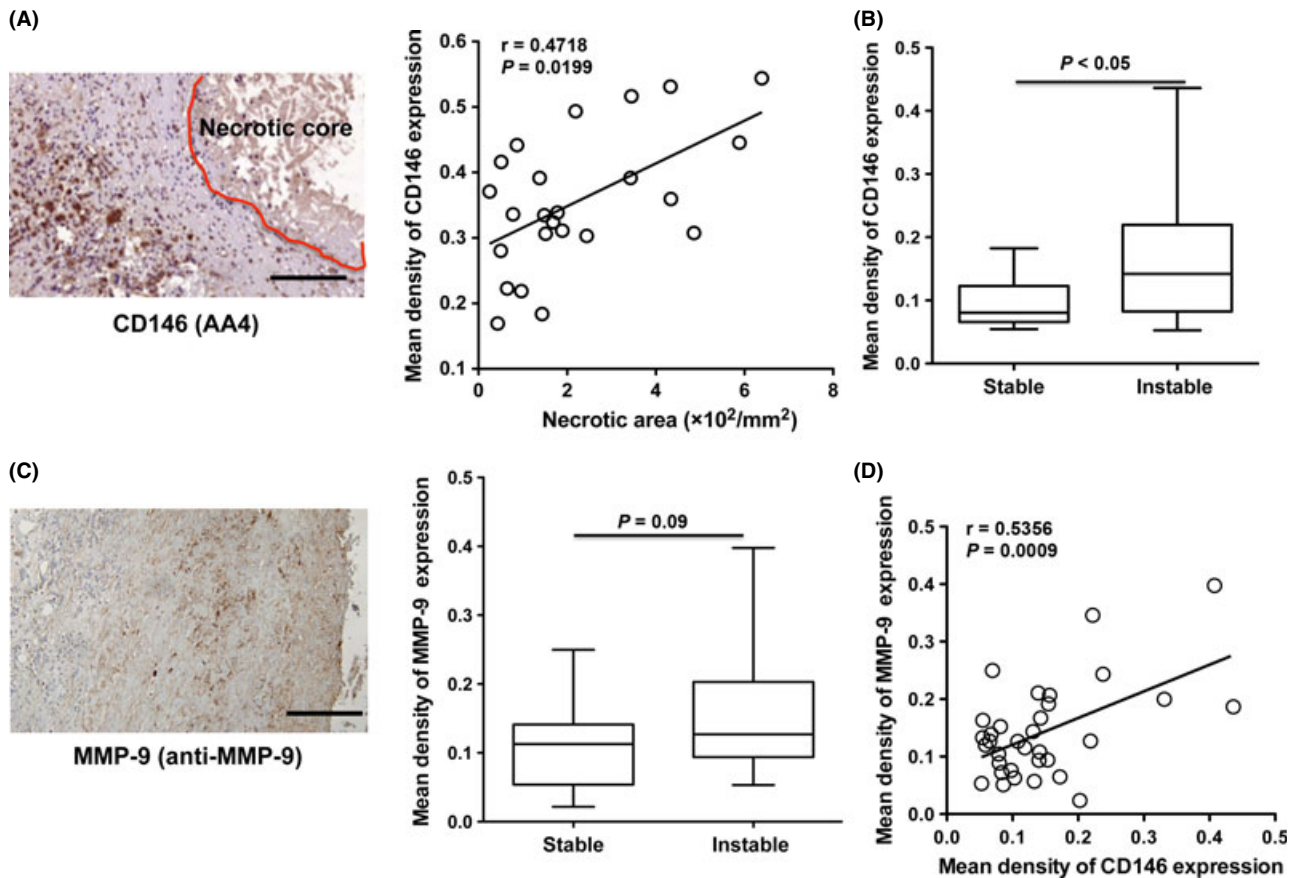


Figure 2 CD146 overexpression is associated with the vulnerability of plaque. (A) CD146 expression is correlated with the necrotic area of plaque (n = 24). (B) CD146 expression is increased in the patients with vulnerable atherosclerotic plaques (n = 24). (C) MMP-9 expression is increased in the patients with vulnerable atherosclerotic plaques (n = 24). (D) The correlation analysis of the CD146 expression and MMP-9 expression in the plaques (n = 40).

shown as the mean ± standard deviation (SD). For comparison of different groups, the nonparametric Mann–Whitney *U* test was employed. For evaluation of the correlations between the sCD146 level and clinical indices, the multiple linear regression and Spearman’s Rank correlation coefficient were employed. We defined the criterion of statistical significance as *P* < 0.05.

Results

CD146 was Upregulated in Human Atherosclerotic Plaques

Using the immunohistochemistry, CD146 expression in atherosclerotic plaques from 40 patients with carotid endarterectomy and five healthy normal carotid arteries was detected. In the atherosclerotic plaques, CD146 was expressed mainly on the smooth muscle cells (α -SMA⁺) (Figure 1A), infiltrated macrophages (CD68⁺) (Figure 1B), and endothelial cells of neovascular blood vessels (CD31⁺) (Figure 1C). While in the health arteries, CD146 was expressed on the smooth muscle cells of the media and perivascular small blood vessels (Figure 1D). Furthermore, the density of CD146 expression was stronger in plaque of neovascular blood vessels than that in the perivascu-

lar vessels of healthy arteries (Figure 1E), implying that the overexpression of CD146 was associated with the atherosclerotic lesions.

CD146 Expression was Associated with the Development of Vulnerable Atherosclerotic Plaques

To test whether CD146 expression was related to the development of vulnerable plaque, the relationship of CD146 expression and necrotic core area in atherosclerotic plaques was analyzed. A positive correlation between the mean density of CD146 expression and the necrotic core area (Figure 2A) was found, indicating the overexpression of CD146 in the plaques. Furthermore, by analyzing the CD146 expression between stable plaques and unstable plaques, we found that the mean density of CD146 expression in the unstable plaques was significantly stronger than that in the stable plaques (Figure 2B), confirming the fact that overexpression of CD146 was associated with the development of vulnerable atherosclerotic plaques.

It has been reported that the MMPs, especially MMP-9, contributes to the development of vulnerable plaque [29]. To further confirm this association of CD146 expression and plaque

progression, we have detected MMP-9 expression in human carotid atherosclerotic plaques. These findings provided the proof of upregulation of MMP-9 in the vulnerable plaques (Figure 2C). In addition, there was a significantly positive correlation between the mean density of CD146 expression and the MMP-9 expression (Figure 2D). These data suggested that the overexpression of CD146 in the plaques might predict the development of vulnerable atherosclerotic plaques.

Soluble CD146 was Increased in the Patients with Atherosclerosis

To test the relationship of CD146 expression and AS inflammation, detecting serum soluble CD146 in 40 health donors and 40 patients with AS by using the sandwich ELISA (Figure 3A) was conducted. Tests showed that level of serum sCD146 was significantly elevated in patients with AS (Figure 3B). Because the soluble CD146 has been reported to originate from the membrane-bound CD146, the correlation of the level of serum sCD146 and expression density of CD146 in the AS plaques ($n = 40$) was further analyzed. We found that there was a significant positive correlation between sCD146 and CD146 expression density (Figure 3C). These data showed that there was a significant positive correlation of CD146 and sCD146 in patients with AS indicating that sCD146 originated from the membrane-bound CD146, might correlate with the inflammation of AS.

Analysis of the Correlation of sCD146 and Clinical Parameters

To further analyze the correlation of sCD146 and inflammatory factors, serum levels of IL-6 and hsCRP, reflecting the activity of inflammation by both, were tested. Both IL-6 and hsCRP were increased in patients with AS compared with that in the normal donors (Figure 4 A,B). In addition, sCD146 had a positive correlation with IL-6 and hsCRP ($P = 0.0044$; $P = 0.005$) (Figure 4C,D), indicating that sCD146 maybe a promising biomarker for monitoring active inflammatory process in AS.

It has been reported that many soluble forms of adhesion molecules, such as sICAM-1 and sVCAM-1, are associated with the risk factors of AS, e.g. age, homocysteine, etc. [30,31]. To investigate whether the level of sCD146 was associated with the risk of developing AS, the correlation of sCD146 and clinical risk factors were analyzed. No such correlation between these factors was found (Table 2), indicating that sCD146 level alone might be an independent risk factor for the development of atherosclerotic inflammation.

sCD146 Level Correlated with the Vulnerability of Plaque

Active inflammation promotes the instability of plaques. To test the association of sCD146 and the plaque instability, sCD146 levels of patients with or without instable atherosclerotic plaques were analyzed. The analysis showed that sCD146 was elevated in patients with instable plaques compared with those with stable plaques, consistent with the finding of increased serum MMP-9 in patients with instable plaques (Figure 5A,B). In addition, sCD146

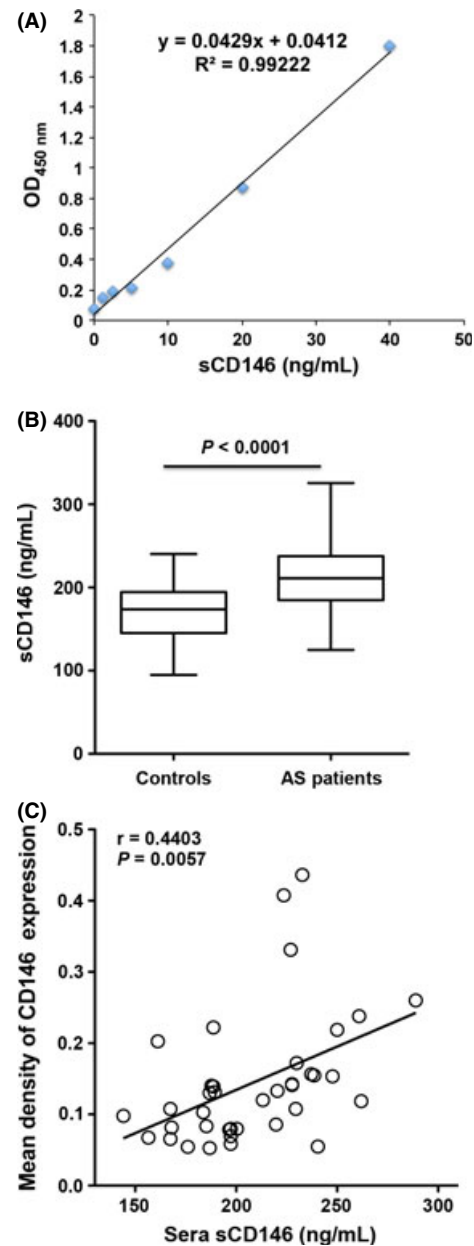


Figure 3 Serum sCD146 is elevated in the patients with AS. (A) Standard curve for the detection of sera sCD146 by sandwich ELISA. (B) sCD146 is increased in the patients with AS ($n = 40$) compared with normal donors ($n = 40$). (C) sCD146 has a positive correlation with the mean density of CD146 expression in the plaques ($n = 40$).

had a positive correlation with the MMP-9 levels in patients with AS (Figure 5C). These data suggested that sCD146 might be a new biomarker for instability of plaques.

Discussion

Many studies have shown that serum soluble CAMs, such as sICAM-1, sVCAM-1, sP-selectin and sE-selectin, are associated with cardiovascular events [32–34]. Monitoring the levels of

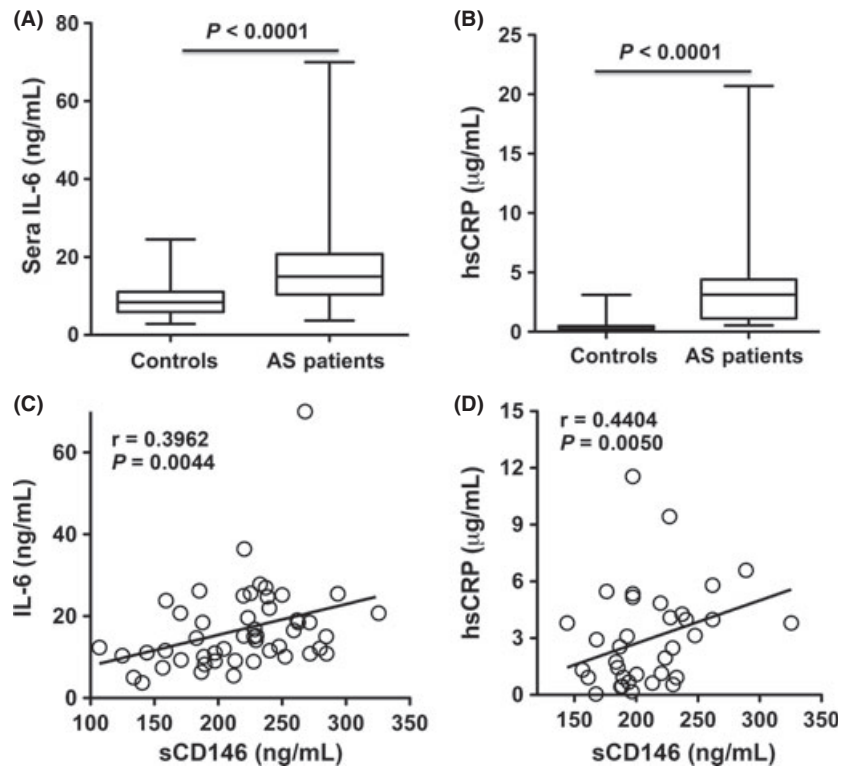


Figure 4 Serum sCD146 in the patients with AS is correlated with the inflammation of disease. **(A)** IL-6 is increased in patients with AS ($n = 40$). **(B)** hsCRP is increased in patients with AS ($n = 40$). **(C)** sCD146 has a positive correlation with IL-6 ($n = 40$). **(D)** sCD146 has a positive correlation with hsCRP ($n = 40$).

Table 2 Correlation analysis between sera sCD146 and various clinical parameters in patients with AS

Sera sCD146	Correlation coefficient	<i>P</i> value
Age (years)	-0.0533	0.7576
Hypertension (years)	-0.0025	0.9886
Diabetes (years)	0.0339	0.8445
TG (mm)	-0.1758	0.3050
TC (mm)	0.0815	0.6367
LDL-C (mm)	-0.0492	0.7755
HDL-C (mm)	0.1156	0.5022

TG, triglyceride; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

these sCAMs can predict the seriousness of vascular events and assist in diagnoses of AS. CD146 has been recognized as an angiogenesis marker and reported to upregulate neovascular endothelial cell [17,18]. In this study, we first found CD146 was upregulated in human atherosclerotic plaques. Its overexpression might predict the intraplaque microvascular angiogenesis within the atherosclerotic lesions. It is considered that the intraplaque neovascularization is an important feature in plaque development and vulnerability, which increases the risk of rupture and cerebral emboli event. In addition, several pathological studies have confirmed that the presence and degree of neovascularization within the plaque is strongly associated with plaque rupture risk [35–37]. Because sCD146 has been reported to come from the membrane-bound CD146, using the sandwich ELISA, we found that sCD146 was elevated in the athero-

sclerotic patients compared with that in normal donors. Furthermore, sCD146 level is higher in patients with vulnerable plaques than in those with stable plaques. sCD146 also correlated with the expression of serum MMP-9, which was associated with plaque vulnerability. Furthermore, sCD146 correlated with the levels of hsCRP and IL-6 in patients with AS, which was indicative of an acute inflammatory process. Our study indicated rather strongly that sCD146 might be a promising biomarker for monitoring the active inflammatory process in AS. Detecting sCD146 levels in patients with AS can be helpful in predicting the seriousness of vascular events and planning for early intervention.

In the process of developing AS, the vulnerability of plaques is the leading cause of disease progression [38–41]. These plaques may rupture, forming thrombus, blocking blood vessel, sending emboli, and causing stroke, tissue ischemia and necrosis. Preventing plaque from becoming vulnerable can reduce the occurrence of serious vascular events. The well-known pathological factors that affect the development of plaque vulnerability are angiogenesis, macrophage infiltration and thinning of the fibrous cap of plaques [42–45]. It has been reported that MMPs, especially MMP-9, play an important role in the plaque fibrous cap thinning process [29,46]. Many studies showed that serum MMP-9 levels were significantly higher in patients with vulnerable plaques than those with stable plaques, indicating the association of MMP-9 and plaque vulnerability. In our study, sCD146 was found to be generated in a matrix metalloproteinase-dependent way [23,47]. In patients with AS, the membrane-bound CD146 on the neovascular endothelial cells in the plaques may be an important source of sCD146. Therefore, serum levels of sCD146 may not only

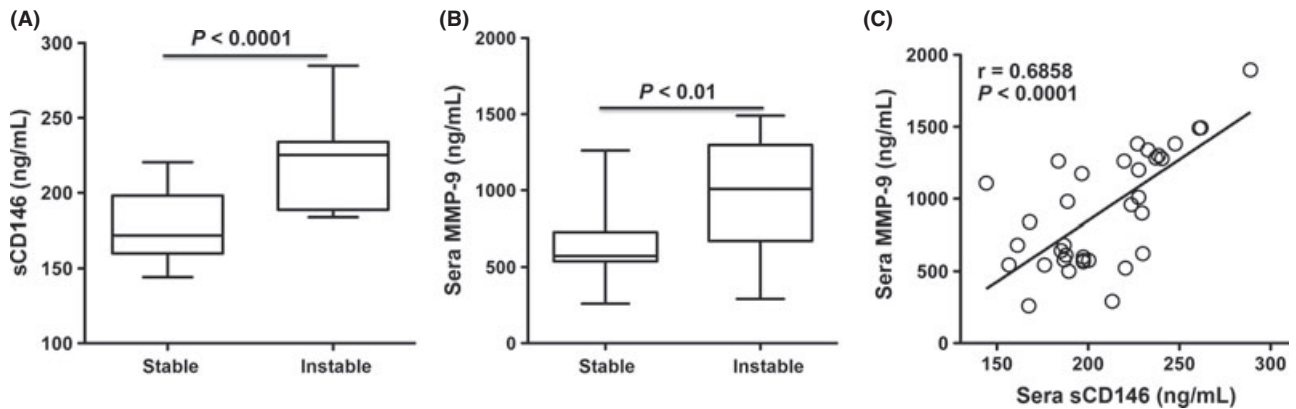


Figure 5 Serum sCD146 is correlated with the vulnerability of atherosclerotic plaques. **(A)** Serum sCD146 is elevated in atherosclerotic patients with vulnerable plaques ($n = 24$). **(B)** Serum MMP-9 is elevated in atherosclerotic patients with vulnerable plaques ($n = 24$). **(C)** sCD146 has a positive correlation with serum MMP-9 ($n = 40$).

reflect the activity of MMPs, but also activity of neovascular endothelial cells. Furthermore, sCD146 correlated with the level of inflammation, such as in the elevation of levels of hsCRP and IL-6. sCD146 may be more sensitive in monitoring the instability of plaque.

Others and we have found that sCD146 is associated with the inflammation activity of various vascular diseases, such as inflammatory bowel disease, multiple sclerosis, diabetic nephropathy, multiple vasculitis, etc. Many studies suggest that sCD146 may have multiple roles in the development of vascular inflammation. A recent report showed that sCD146 has pro-angiogenic property and induces angiogenesis in animal models of ischemic limb necrosis [48]. In our recent study, we found that sCD146 may be an inflammatory factor inducing cell adhesion molecules such as ICAM-1, VCAM-1 expression in endothelial cells and facilitating the transendothelial migration of inflammatory cells, thereby promoting the development of inflammation [23].

What is then the exact role of sCD146 in AS development? Some studies have been suggested that serum sCD146 may be associated with the migration of monocytes into the plaques [49,50]. sCD146 may bind with an unknown ligand on the monocytes, and promote the interaction of monocytes and the activated endothelial cells, thereby facilitating the transmigration of monocytes to the plaques, and promoting the plaque instability and

causing AS progression. In addition, sCD146 may be associated with plaque neovascularization and indirectly or directly promote the plaque instability due to its pro-angiogenic and pro-inflammatory properties. Further studies of the role and mechanism of sCD146 during the process of the development of AS are needed.

Conclusion

Our study has provided the evidence for the first time that CD146, a new adhesion molecule, is associated with the progression of atherosclerotic lesions in the diseased vessel wall. Testing for serum sCD146 might predict the degree of inflammatory activity and development of vulnerable atherosclerotic plaque.

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Conflict of Interest

The authors declare no conflict of interest.

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