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Original Article

The Correlation between Increased Expressions of NLRP3 Inflammasome Components in Peripheral Blood Mono-Nuclear Cells and Plaque Vulnerability in Human Carotid Atherosclerosis

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Abstract

Background: We aimed to determine whether NLRP3 inflammasomes in peripheral blood mononuclear cells (PBMC) were associated with carotid plaque instability in carotid atherosclerosis patients.

Methods: Consecutive 38 carotid atherosclerosis with vulnerable plaques, 22 carotid atherosclerosis with stable plaques, and 40 healthy subjects with no carotid or coronary artery stenosis were enrolled. They were referred to the Second Hospital of Dalian Medical University from 2013-2019. Carotid plaques were evaluated by modified plaque vulnerability risk score (MPVRS) and pathological assessment. The mRNA and protein expression of NLRP3 inflammasome components in PBMC were determined by quantitative real time PCR and Western blot analysis or ELISA.

Results: When consecutive study subjects undergoing carotid endarterectomy were divided into stable (≤4) and unstable (>4) plaque groups according to the MPVRS, the unstable plaque group had significantly raised mRNA and protein expression of NLRP3 inflammasome components in PBMC as compared with the stable plaque group and healthy subject group. Furthermore, subjects with higher NLRP3 protein expression in PBMC had greater incidence of cerebrovascular events.

Conclusion: Increased NLRP3 inflammasome components in PBMC is associated with instability of human carotid atherosclerotic plaques, suggesting NLRP3 inflammasome as a potential biomarker for monitoring carotid plaque instability.

Keywords: Carotid atherosclerosis; NLRP3 inflammasome; Mononuclear cells; Plaque stability

Introduction

Rupture of carotid atherosclerotic plaques is the primary cause of ischemic cerebrovascular events (1,2), which consists of almost half of stroke patients in the Chinese population (3). Plaques at high risk of rupture is referred as the unstable or vulnerable plaques, which is characterized by a proinflammatory state. Evidence shows that macrophage infiltration not only plays important

roles in the initiation and development of atherosclerosis, but also contributes to the destabilization of plaques (4-6). However, identification of plaque at risk of rupture remains a great challenge for both clinical and basic researchers (2,7). Unstable plaques have structural and functional characterization including macrophage infiltration and intraplaque neovascularization. Macrophages infiltration maintains a proinflammatory microenvironment and promotes formation of unstable plaques. Intraplaque neovascularization and hemorrhages play critical roles in the transition of plaques from the stable state to unstable state and their rupture. Currently, pathological analysis of atherosclerosis plaques still remains the gold standard for evaluation of carotid plaque stability, however, due to its invasiveness, it is limited to be widely accepted in clinical practice. Extensive studies have been recently focusing on alternative methods for the evaluation of the carotid plaque vulnerability (8-10). Carotid ultrasound, a noninvasive and non-ionizing imaging technique, provides valuable information to distinguish vulnerable from non-vulnerable plaques based on sonographic features such as stenosis, plaque echogenicity, echolucency, neovascularization, ulceration (11,12). Circulating cytokines such as tumor necrosis factor, fractalkine, IL-6 have been suggested to be biomarkers of carotid plaque vulnerability (13,14). Although the advancement in both histopathologic and noninvasive techniques are being developed to identify vulnerable plaques, the wide usage of these techniques is limited by the risk of invasiveness, dependence on needs of cost instruments, radiation or contrast reagents, and/or low accuracy or reproducibility. Alternative methods on the evaluation of the carotid plaque vulnerability (9,13) have been extensively studied in recent decades.

The expression of the NLRP3 inflammasome and its downstream inflammatory cytokines are higher in PBMC of patients with acute coronary syndrome than healthy controls, and the expression level is positively associated with the severity of acute coronary syndrome (15, 16). Shi et al recently have shown greater expression of components of the NLRP3 inflammasome signaling pathway components within vulnerable human carotid atherosclerotic plaques than those of stable plaques (17). The NLRP3 inflammasome may serve as an index of the stability of atherosclerotic plaques.

In this study, we measured expression levels of NLRP3 inflammasome components in PBMC in patients with carotid atherosclerotic plaques and assessed the stability of plaques by pathological characteristics and carotid sonography. We investigated the association of the expression level of NLRP3 inflammasome components in PBMC with macrophage infiltration and neovascularization of carotid plaques and NLRP3 level in PBMC could predict the occurrence of postoperative cerebrovascular events.

Methods

Study subjects

Sixty consecutive patients with ipsilateral symptoms in last 6 months (stroke, transient ischemic attack or amaurosis fugax) and internal carotid stenosis ≥70%, referred to the Second Hospital of Dalian Medical University, China were scheduled for carotid endarterectomy (CEA) surgery between October 2013 and December 2019 enrolled. The symptoms were evaluated by a neurologist and the stenosis was measured by duplex ultrasonography. Forty healthy subjects who had no coronary and carotid atherosclerotic plaques were recruited as normal control.

All subjects underwent clinical and biochemical examinations. Exclusion criteria were prior cerebral infraction (within 20 days), carotid occlusion, vasculitis, acute infection, chronic hepatic and renal dysfunction, malignancy, unstable angina or myocardial infarction within 6 months, prior radiation therapy to the neck, treatment with immunomodulating drugs or oncological disease.

The study was approved by the Ethics Committee of the Second Hospital of Dalian Medical University. Procedures followed institutional guidelines, and written informed consent was obtained from all participants.

Plaque assessment

In addition to naked eye observation and hematoxylin and eosin (HE) staining, carotid plaques were evaluated by Modified Plaque Vulnerability Risk Score (MPVRS) as described (11,18) with modification: echogenicity (1=hyperechogenicity, 2=intermediate echogenicity, 3=hypoechogenicity or echolucency), texture (0=homogeneous, 1=heterogeneous), surface (0=smooth, 1=irregular, 2=rupture or ulcer), and eccentricity (1=mild eccentricity, 2=severe eccentricity). The unstable plaques were those had ≥4 MPVRS score with either naked eye and/or histopathological observation showing incomplete endothelium, plaque surface rupture, erosion, ulcers or bleeding, a large number of mononuclear macrophages, de novo vasculogenesis, thin fibrous cap or lipid core larger than 40% of plaque volume; the stable plaques were those without above observation. All ultrasound examinations were performed by two sonographers who were blinded to patients' clinical details.

Isolation of peripheral blood mononuclear cells

Human PBMC were isolated by Ficoll-Hypaque density-gradient centrifugation. The purity of isolated monocytes was checked with anti-CD14 by flow cytometry. Isolated monocytes were cultured in medium RPMI-1640 with 10% fetal bovine serum in an incubator at 37 ℃ with 5% CO_2 as described (16).

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) were performed as described previously (17). Briefly, cellular total RNA from PBMC was extracted using RNA extraction Kit (Omega Bio-Tek, Norcross, GA). Isolated mRNA was reverse transcribed to cDNA with Prime Script TMRT reverse transcriptase (RR047A, Takara, Dalian, China). One µl of cDNA was used for real-time polymerase chain reaction (SYBR Green qPCR Kit, Takara, Dalian, China) under the following conditions: 95 °C for 30 seconds, followed by 40 cycles at 95 °C for 5 seconds and 60 °C for 30 seconds. Primer sequences used in this study are described previously (17). The gene expression relative *β-actin* was analyzed using the comparative ΔCt method as previously described (13). Specific primers for real-time RT-qPCR are presented in Table 1.

Table 1: Specific primers for real-time RT-qPCR

Genes Primers	Sequence (5'-3')
NLRP3(f)	AAAGCCAAGAATCCACAGTGTAAC
NLRP3 (r)	TTGCCTCGCAGGTAAAGGT
ASC (f)	GGATGCTCTGTACGGGAAGG
ASC(r)	CGCATCTTGCTTGGGTTG
Caspase1(f)	AGGCATGACAATGCTGCTACAA
Caspase1(r)	TGTGCAAATGCCTCCAGCTC
IL-1β(f)	TCGCCAGTGAAATGATGGCTTA
IL-1β(r)	GTCCATGGCCACAACAACTGA
IL-18(f)	GACCTTCCAGATCGCTTCCTC
IL-18(r)	GATGCAATTGTCTTCTACTGGTTC
β-actin(f)	CCATCGTCCACCGCAAAT
β-actin(r)	GCTGTCACCTTCACCGTTCC

Note: (f), forward primers, (r), reverse primers

Immunoblotting analysis

Immunoblotting analysis was performed as described earlier (17). Briefly, cellular total proteins from PBMC were extracted using a Protein Isolation Kit (Omega Bio-Tek, Norcross, GA). Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL). Equal protein samples were run on 10% SDS gels and transferred on nitrocellulose membranes. The protein samples were incubated with primary antibodies against GAPDH(1:20,000 dilution, 60004-1-Ig, Proteintech), NLRP3(1:1000 dilution, 19771-1-AP, Proteintech) , ASC(1:200 dilution, sc-33958, Santa Cruz Biotechnology), Caspase 1 (1:200 dilution, LS-B3394, LifeSpan BioSciences Inc), IL-1β(1:2000 dilution, 66737-1-Ig, Proteintech),IL-18(1:2000 dilution, 60070-1-Ig, Proteintech). Membranes were incubated with indicated primary antibodies overnight at 4 °C with gentle shaking. After incubation with proper secondary antibodies for 1 hr at room temperature, blotted proteins were visualized with an enhanced chemiluminescence detection system (ECL; Advansta, Menlo Park, CA).

ELISA

Serum IL-1β and IL-18 levels were determined using ELISA kits (eBioscience, Tianjin, China) following manufacturer's instruction as described previously (19). Briefly, 50 μl of plasma sample in each well were incubated for 2 h, following with 200 μl conjugate for 2 h, then reacted with substrate for 20 min. Absorption was read at 450 nm after incubation with stop solution for 30 min.

Histological and immunohistochemistry Studies

The tissue samples were removed and immersed in freshly prepared 4% paraformaldehyde for 24 h and then embedded in paraffin for HE staining and immunohistochemistry as described previously (17,20). Slides were boiled for epitope retrieval in citrate buffer for 15 min before incubation with mouse anti-CD68 monoclonal antibody (1:500, GB113150, Servicebio) or anti-CD34 antibodies (1:500, GB111693, Servicebio) overnight at 4 °C. The immunohistochemistry reactions were developed with diaminobenzidine. Quantitative analysis of macrophage infiltration was performed by counting CD68-positive cells in five randomly fields under a light microscope at ×200 magnification. Microvessel density or neovascularization was determined by counting the number of CD34-positive endothelial cell or endothelial cell clusters in five randomly fields. The average of five determinants was calculated. Stained sections were imaged by Leica DM4000.

Clinical follow-up

Postoperative neurological and cardiovascular ischemic events (death, stroke, transient ischemic attack, amaurosis fugax, angina, acute myocardial infarction) with a mean follow-up time of ± months were conducted with telephone call or office visit. All of the death cases were verified against the local or national government population register.

Statistical analysis

Statistical analysis was performed using the SPSS, version 21.0 (IBM Corp., Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation. The Students t-test or one-way ANOVA was used if continuous variables were normally distributed, and the Fisher's exact test was used for categorical variables. Kaplan-Meier survival analysis was used to determine the statistical significance of difference during follow up. $P<0.05$ was considered statistically significant.

Results

Characteristics of study subjects

Baseline clinical characteristics for the control, carotid atherosclerosis with stable and unstable plaques are shown in Table 2. The carotid atherosclerosis group either with stable or unstable plaques showed a higher histories of hypertension, diabetes mellitus, and cerebrovascular disease, and higher age, systolic blood pressure, plasma concentrations of triglycerides, as well as lower plasma concentration of HDL-C and Apo-

A compared to the control group (all $P<0.05$). There were no differences among the three groups with respect to the sex, incidence of smoking or drinking, diastolic blood pressure, LDL-C, Apo-B, white blood cells count, mononuclear cells, serum urea, and serum creatinine.

Table 2: Baseline clinical characteristics of the control, stable plaque group and unstable plaque group

Variable	*Control (n=40)*	*Stable plaque group (n=22)*	*Unstable plaque group (n=38)*
Age (yr)	61.13±9.52	66.22±7.42[a]	66.05±8.09[a]
Male sex, n (%)	18 (45)	19 (86.4)[a]	32 (84.2)[a]
Smoking, n (%)	8 (20)	10 (45.4)	22 (57.9)
Drinking, n (%)	5 (12.5)	6 (27.3)	6(15.8)
Systolic Blood pressure, mmHg	129.23±14.99	142.91±15.72[a]	141.61±20.27[a]
Diastolic Blood pressure, mmHg	79.75±10.65	82.77±9.5	81.21±11.43
Medical history, n (%)			
Hypertension	14 (35)	19(86.4)[a]	28(73.7)[a]
Type 2 diabetes	7 (17.5)	13(59.1)[a]	20(52.6)[a]
Symptomatic	0 (0)	8 (36.4)	27(71.1)[ab]
Clinical Parameters			
White blood cells ($\times10^9$/L)	6.30±1.32	6.25±1.48	6.76±1.50
Mononuclear cells ($\times10^9$/L)	0.38±0.15	0.46±0.32	0.42±0.15
BUN (mmol/L)	5.48±1.63	6.20±1.76	5.61±1.82
Creatinine (umol/L)	70.43±12.97	74.33±9.48	73.19±10.87
Uric Acid (umol/L)	309.23±74.34	312.50±56.53	338.75±98.68
Triglycerides (mmol/L)	1.57±1.03	2.19±0.87[a]	2.02±0.9[a]
HDL-Cholesterol (mmol/L)	1.16±0.26	1.04±0.23	0.97±0.24[a]
LDL-Cholesterol (mmol/L)	2.61±0.91	2.26±0.81	2.60±0.76
Apo-A1 (g/L)	1.32±0.17	1.24±0.18	1.21±0.20[a]
Apo-B (g/L)	0.91±0.17	0.84±0.24	0.92±0.23

a $P<0.05$ *vs* control, b $P<0.05$ *vs* stable plaque, analysis of variance (ANOVA), Newman–Keuls test

Ultrasonographic analysis of carotid atherosclerotic plaques.

Ultrasonographic analysis of stable and unstable carotid atherosclerotic plaques are shown in Fig. 1. Unstable plaque has hypoechogenicity, heterogeneous texture, severe eccentricity and smooth surface. Stable plaque has hyperchogenicity, heterogeneous texture, mild eccentricity and smooth surface.

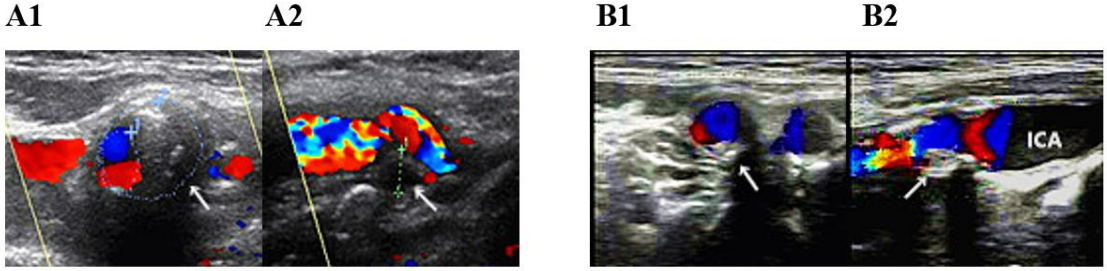

Fig. 1: Ultrasonographic analysis of carotid atherosclerotic plaques.

A1 and A2 demonstrates a case of unstable plaque of severe carotid stenosis with white arrow.(A1) Longitudinal view of the carotid plaque on color Doppler ultrasound. (A2) Cross-sectional view of the carotid plaque on color Doppler ultrasound. This unstable plaque with MPVRS score 6, showing hypoechogenicity(score=3), heterogeneous texture(score=1), severe eccentricity(score=2), smooth surface(score=0).
B1 and B2 demonstrates a case of stable plaque of severe carotid stenosis with white arrow.(B1) Longitudinal view of the carotid plaque on color Doppler ultrasound. (B2) Cross-sectional view of the carotid plaque on color Doppler ultrasound. This unstable plaque with MPVRS score 3, showing hyperechogenicity(score=1), heterogeneous texture(score=1), mild eccentricity(score=1), smooth surface(score=0)

Pathological characteristics of stable and unstable carotid plaques

Representative HE staining of stable and unstable carotid atherosclerotic plaques obtained from CEA surgery are shown in Fig. 2. Plaques with vulnerable phenotypes showed larger core filled with more cholesterol clefts, thinner fibrous cap with increased formation of intraplaque hemorrhage, compared to those in stable plaques.

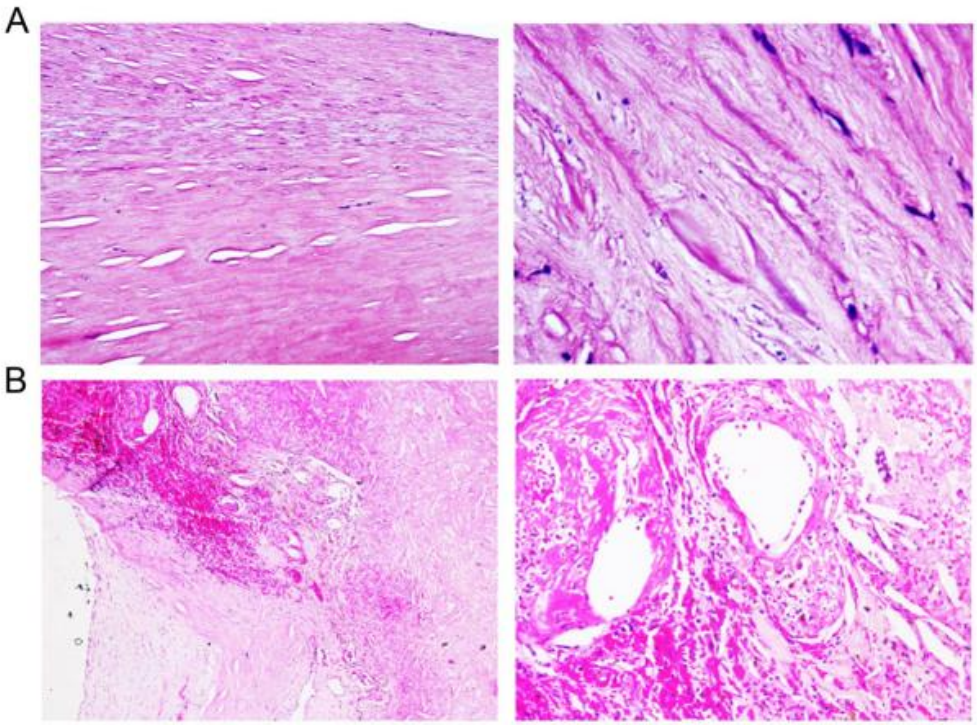

Fig. 2: Representative histological images of carotid plaques, stained with Hematoxylin and eosin. (**A**) A stable carotid plaque. No obvious erosion, ulcer, hemorrhage or blood clot, granulocytic or cap macrophage infiltration or plaque rupture was observed. Magnification: left, ×40; right, x200. (**B**) An unstable carotid plaque. Obvious neovascularization, some with blood clot, clustered lymphocyte infiltration, intraplaque hemorrhage, or cholesterol clefts were observed. Magnification: left, ×40; right, x200

Immunohistochemical staining showed that unstable plaques had greater macrophage infiltration and increased number of endothelial or endothelial clusters, assessed by CD68 and CD34 respectively in Fig. 3, indicating unstable carotid plaques had high active inflammatory responses and more neovascularization formation compared to the stable carotid plaques.

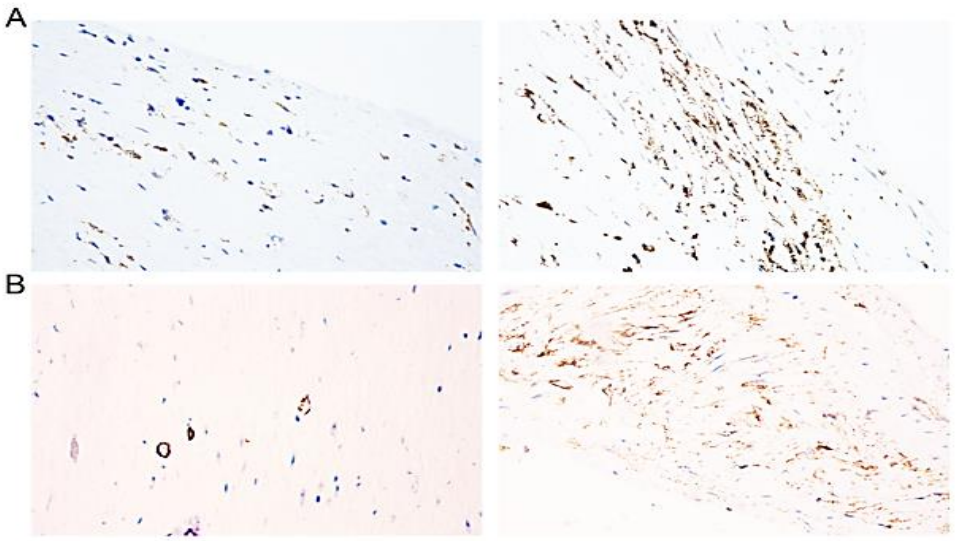

Fig. 3: Macrophage infiltration and neovascularization within carotid plaques. (**A**) A representative of immunohistochemical staining with CD68. A stable carotid plaque (left), compared to unstable carotid plaques (right), less CD68-positive macrophages were observed. Magnification, x200. (**B**) A representative of immunohistochemical staining with CD34. A stable carotid plaque (left), compared to unstable carotid plaques (right), fewer neovascularization was observed. Magnification, x200

Expression of NLRP3 inflammasomes in PBMCs

Plasma concentrations of IL-1β and IL-18 had no statistically significant difference between unstable and stable subjects (Fig. 4).

Significantly higher mRNA expression levels of NLRP3, IL-1β, and IL-18 in PBMC from patients with unstable plaques was observed compared to those with stable plaques and normal subjects (Fig. 5).

Consistently, the protein expression levels of NLRP3, IL-1β, and IL-18 were significantly higher in patients with unstable plaques compared to those with stable plaques and normal subjects (Fig. 6).

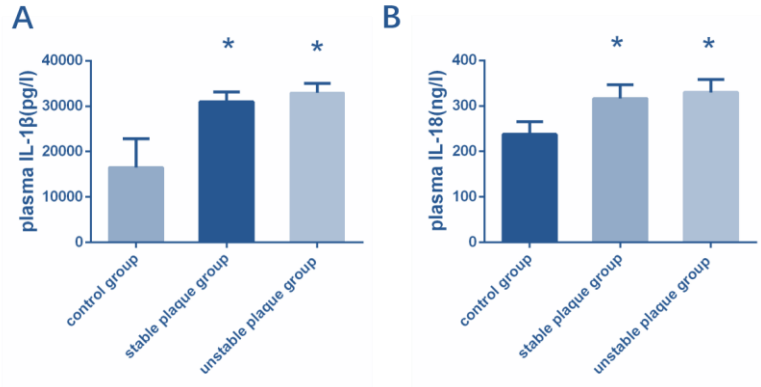

Fig. 4: Plasma level of IL-1β (A) and IL-18 (B) in healthy subjects and patients with stable and unstable carotid plaques. Plasma levels of IL-1β (**A**) and IL-18 (**B**) were determined by ELISA assay. Although the plasma IL-1β and IL-18 level increased in both the stable (n=22) and unstable (n=38) plaque groups compared to the control group (n=40), but no statistically significant difference between the stable and unstable plaque groups was observed. **P*<0.05 vs. control, analysis of variance (ANOVA), Newman-Keuls test

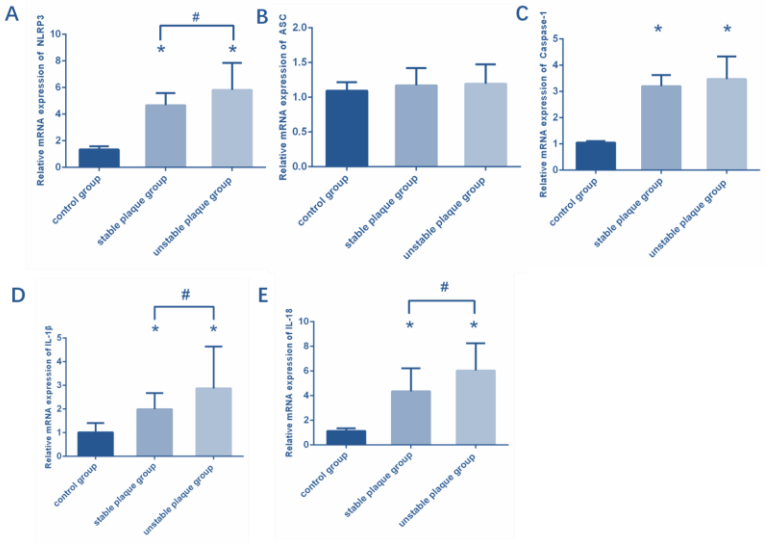

Fig. 5: The relative mRNA expression of NLRP3 inflammasome components in PBMC from healthy subjects and patients with stable and unstable carotid plaques. The relative mRNA expression levels of NLRP3 (**A**), ASC (**B**), Caspase-1 (**C**), IL-1β (**D**), and IL-18(**E**) were determined by real-time quantitative reverse transcription PCR. * *P*<0.05 *vs* control, # *P*<0.05 *vs* stable plaque, analysis of variance (ANOVA), Newman–Keuls test

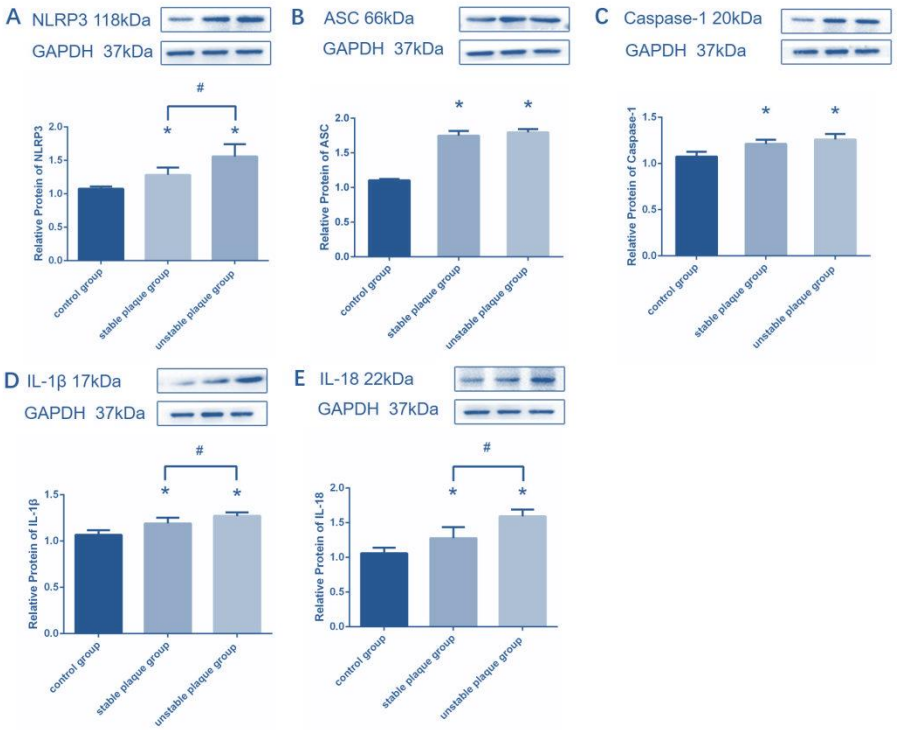

Fig. 6: Relative protein expression of NLRP3 inflammasome components in PBMC from healthy subjects and patients with stable and unstable carotid plaques. The band density of NLRP3 (**A**), ASC (**B**), Caspase-1 (**C**), IL-1β (**D**), and IL-18(**E**) were quantified by ImageJ from two independent experiments. * *P*<0.05 *vs* control, # *P*<0.05 *vs* stable plaque, analysis of variance (ANOVA), Newman–Keuls test

NLRP3 protein expression in PBMC in prediction of cerebrovascular events

Subjects with higher level of NLRP3 protein expression in PBMC had an increased incidence of cerebrovascular events compared to the middle level and lowest level of NLRP3 in PBMC (Fig. 7).

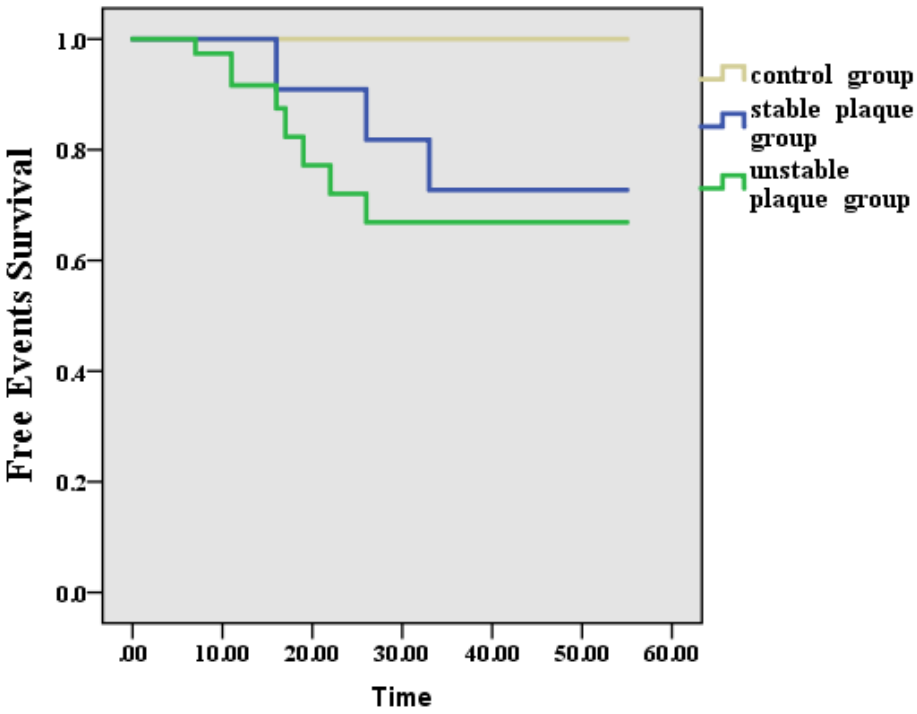

Fig. 7: Free from major adverse cerebrovascular events in healthy subjects and patients with carotid plaques. Patients were followed up for a median of 33.99 months. Free from major adverse cerebrovascular events rate (%) with low (orange), middle (blue) and high (green) levels of NLRP3 protein expression was determined by the log-rank test. Results of the Kaplan–Meier survival analysis are shown. The composite outcome of all-cause mortality and non-fatal cardiovascular and cerebrovascular events occurred in 8（21.1%）unstable plaque group patients and in 3（13.6%）stable plaque group patients and no events happened in control group during follow-up. Patients with unstable carotid plaques had higher adverse cerebrovascular events than patients in stable groups (log-rank test, $\chi 2=12.5$, $P<0.05$)

Discussion

The present study demonstrated the greater expression of major NLRP3 inflammasome components in PBMC in patients with unstable carotid plaques compared to those with stable plaque patients and healthy subjects. The mRNA expression level of NLRP3 components in PBMC correlated positively with the amount of macrophage infiltration and neovascularization of carotid plaques. We also found that patients with higher NLRP3 protein expression in PBMC had an increased risk of cerebrovascular events during the 33.9 months follow-up period. Our results indicate that the expression of NLRP3 inflammasomes in PBMC correlates positively with the instability of carotid atherosclerotic plaques.

Activation of NLRP3 inflammasomes, the intracellular pattern recognition receptors, has been demonstrated to be a major contributor in the process of atherogenesis (21,22). Gene silencing of NLRP3 in Apo-E deficient mice decreases the amount of macrophages and lipid accumulation and increases content of smooth muscle cells and collagen in plaques, leading to the stabilization of

atherosclerotic plaques (23,24). We have recently found that the expression of NLRP3 inflammasome components including NLRP3, ASC, caspase-1, IL-1βand IL-18 are remarkably increased within plaques in patients with unstable carotid atherosclerosis, compared to those patients with stable carotid atherosclerosis and healthy mesenteric arteries (17), and in this study, we showed that the gene and protein expression of NLRP3 inflammasome components was significantly increased in patients with unstable carotid plaques compared to those with stable plaques and healthy subjects (Fig. 5, 6). However, there were no significant difference in the plasma IL-1β and IL-18 between patients with unstable and stable carotid plaques (Fig. 4).

The reason for the poor association of plasma and PBMC levels of IL-1β and IL-18 was not clear. A likely explanation would be that IL-1β and IL-18 secreted from PBMC into the circulating pool were not great enough to distinguish between patients with unstable and stable plaques. Of note, the monocyte is only a small portion of circulating blood cells in patients with unstable and stable plaques, therefore, plasma concentrations of IL-1β and IL-18, downstream cytokines of NLRP3 inflammasomes did not reflect well with the carotid plaque vulnerability (Fig. 4).

Identification of potential biomarkers for high-risk or vulnerable carotid plaque has been clinically important (5,25). Morphologic features of carotid plaques assessed by imaging methods can be obtained to evaluate plaque vulnerability, but with limitation of wide usage (12,26,27). MPVR score calculation is an interesting method to evaluate the risk of carotid plaques by plaque echogenicity, texture, surface and eccentricity. Although these considerable efforts, identification of a reliable method to evaluate carotid plaque stability remains a challenging task. In the current study, we evaluated the expression level of NLRP3 inflammasomes in PBMC to estimate the stability of plaques, we found that NLRP3 inflammasomes in PBMCs were positively correlated with plaque macrophage infiltration and neovascularization, and also associate with the risk of cerebrovascular events during the follow-up period, indicating activity of NLRP3 inflammasomes in PBMC reflected pathological characteristics and prognosis of atherosclerotic carotid plaques, which might be employed to evaluate the stability of carotid plaques.

The present study had limitation on its potential bias on selection of study subjects, which limited to patients for stenosis greater than 70% in our hospitals, and a relatively small number of patients enrolled in each subgroup. This study was also limited by lack of dynamic changes of expression of NLRP3 inflammasome components in PBMC during the follow-up period. A larger cohort of subjects with longer follow-up study needs to confirm the current observation.

Conclusion

The higher gene and protein expression of certain NLRP3 inflammasome components in PBMC correlated with carotid plaque instability. Analysis of the NLRP3 inflammasome expression in PBMC combined with ultrasonic and other imaging and non-imaging techniques may identify subjects with high-risk of carotid atherosclerotic plaques.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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