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Original Article

The correlation between calcification in carotid plaque and stroke: calcification may be a risk factor for stroke

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Abstract: Background: There are few studies on the relationship between calcified plaques and stroke by ultrasound. We investigated the association between calcification in carotid plaque and stroke by analyzing the different characteristics of calcification and neovascularization distribution in heterogeneous plaques by both two-dimensional and contrast-enhanced ultrasound (CEUS). Methods: A total of 69 patients who were about to undergo carotid endarterectomy were selected between January 2016 and December 2017. Thirty-eight patients with cerebral ischemia were placed in the symptomatic group (amaurosis, TIA and no disability in the previous 6 months), and the other 31 patients, who were asymptomatic, were placed in the asymptomatic group. Two-dimensional ultrasound and CEUS were used to detect the calcification distribution characteristics and neovascularization in the plaques of all subjects. The differences of the calcification location, shape, quantity, and enhancement in the plaques were compared between the two groups. Results: There was no significant difference in calcification location between the two groups ($P > 0.05$). The symptomatic group had more nodular calcification but less strip calcification compared with the asymptomatic group (both $P < 0.05$). There was no significant difference in mixed morphologic calcification between the two groups ($P > 0.05$). The symptomatic group had a higher calcification quantity ($P < 0.05$). The symptomatic group exhibited more fibrous cap fracture and intra-plaque hemorrhage in H&E staining ($\chi^2 = 17.133$, $P < 0.001$, $\chi^2 = 10.003$, $P = 0.003$) and higher CD31 expression ($t = 7.584$, $P = 0.000$). Conclusions: The quantity and shape of the calcification, and the presence of neovascularization adjacent to the calcification have certain effects on the stability of plaques. Multiple calcifications, nodular calcification, and neovascularization near calcification may cause plaque rupture and therefore might be risk factors for stroke. Our results suggest that the joint use of two-dimensional ultrasound and CEUS can provide comprehensive information on plaques to assess their stability.

Keywords: Calcification, distribution, stability, neovascularization, stroke

Introduction

Cerebrovascular diseases are associated with the highest rate of morbidity and disability in the world, seriously threatening human life [1]. Carotid atherosclerosis plaques are an important pathological basis and risk factor for cerebrovascular disease. Thromboembolism from vulnerable plaques causes cerebrovascular accident, particularly cerebral infarction [2]. If the risk factors of cerebral apoplexy can be found early on in the event of cerebral ischemia, the occurrence of stroke and cerebral infarction could be avoided and could provide an objective basis for active clinical prevention. In clinical work, the incidence of the carotid heterogeneous plaque with calcification is more common than hypoechoic and hyperechoic plaques. The calcification in plaque is usually considered a sign of stability [3]. Now high resolution MRI can detect calcification and other morphological characteristics in plaque, but it cannot find neovascularization in plaque, and it is also expensive and inconvenient for repeated examinations. Conventional two-dimensional ultrasound can roughly evaluate plaque instability according to its echo and morphological characteristics, but it cannot show the neovascularization in the plaque. Contrast-enhanced ultrasound (CEUS) may constantly and dynamically observe neovascularization in atherosclerotic plaques, and noninvasively evaluate their vulnerability. Moreover, it has been reported that hypoechoic plaques and ulcerative plaques are

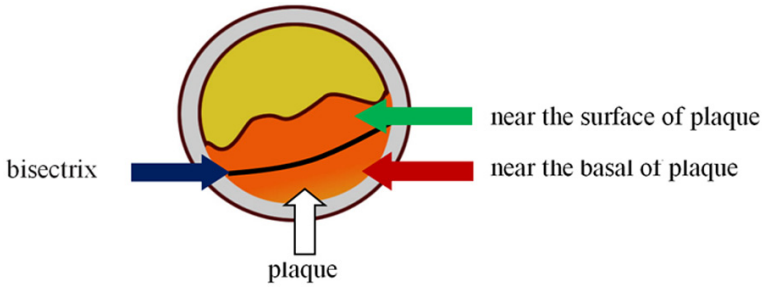

Figure 1. Schematic presentation of the plaque short axis. The white arrow indicates plaque. The blue arrow indicates the bisectrix of plaque, the red arrow indicates near the basal of the plaque, and the green arrow indicates near the surface of the plaque.

vulnerable plaques [4], but few studies have looked at the stability of calcification in plaques. Does calcification play an important role in evaluating the stability of plaque? Is calcified plaque closely related to stroke? We conducted this study on these issues to evaluate the correlation between different characteristics of calcification and neovascularization distribution in heterogeneous plaques and stroke.

Patients and methods

Patients

This study included a total of 69 patients who were about to undergo carotid endarterectomy (CEA) in the Beijing Tiantan Hospital Affiliated to Capital Medical University between January 2016 and December 2017. There were 61 males and 8 females. Among them, 38 patients with cerebral ischemia were placed in the symptomatic group (amaurosis, TIA and no disability in the past 6 months), and the other 31 cases made up the asymptomatic group. Informed consent was obtained from all patients. Inclusion criteria were as follows: in the symptomatic group, we confirmed the ischemic infarct in the unilateral anterior circulation area by CT or MRI examination and defined by amaurosis, TIA, and non-disabling in the 6 previous months, the total volume of calcification was less than 1/2 of the plaque, and the calcification posterior sound shadow did not affect the ultrasonic observation. Exclusion criteria were as follows: pure calcified plaque, calcification on the plaque surface or the basal part of anterior wall was serious, which affected the observation according to the rear echo attenuation effect, patients who were unconscious and could not perform the examination, heart, lung, or renal insufficiency, atrial fibrillation, and contrast agent allergy.

Instruments and methods

An Aplio 500 ultrasonic diagnostic apparatus (Toshiba, Tokyo, Japan) equipped with 11-L4 linear-array transducer and 4-11 MHz frequency, and SonoVue were used in the study. All results were examined by two doctors experienced in ultrasonic diagnostics.

Two-dimensional conventional ultrasound imaging of carotid plaques

When performing the ultrasonography, the subject's head was slightly back and to the opposite side. The location, number and shape of the calcification in the plaque were respectively observed on the long axis and short axis sections of the carotid artery. Under conventional two-dimensional gray-scale ultrasound, the characteristics of the plaques were described according to the modified Gray Weale classification [5] and the division method of the European Carotid Plaque Research Group with the echo characteristics of plaque [6]. The plaque echogenicity was classified into hypo echo (echo slightly higher than the blood), iso echo (similar to the sternocleidomastoid echo), heterogeneous echo (calcification or extremely low echo can be seen inside the plaque, the area of which was less than 50%) and hyperecho (higher than the sternocleidomastoid echo). Based on Ningbin et al.'s [7] method, the plaque's short axis was observed and equally divided the plaque into 2 parts in this study (**Figure 1**)-near the basal and near the surface. The calcification positions of the plaque (**Figure 2A-C**) were recorded.

Contrast-enhanced ultrasound imaging of carotid plaque

The operator placed the probe on the surface of the body and chose a clear region of the plaque. Before examination, the patients were asked to avoid body movement, swallowing, and excessive breathing. The ultrasound contrast mode and double-width real-time display were turned on, simultaneously showing the two-dimensional gray-scale interface and ultrasound contrast interface. The contrast-enhanced ultrasound imaging application included a low mechanical index (0.10-0.12) to avoid early bubble destruction and harmonics with

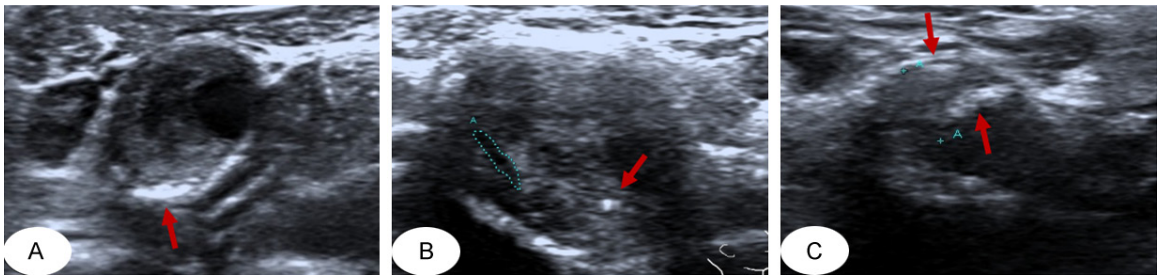

Figure 2. Representative two-dimensional conventional ultrasound images showing different locations of plaque calcification. A. The case is a 65-year-old man. The arrow is the coarse calcification near the basal of plaque in the left carotid artery bifurcation. B. The case is a 58-year-old man. The arrow is the coarse calcification near the intima and the streak-like calcification near the basal respectively of the plaque in the right carotid artery bifurcation. C. The case is a 73-year-old man. The arrow is the small spot calcification near the intima of plaque in the right carotid artery bifurcation.

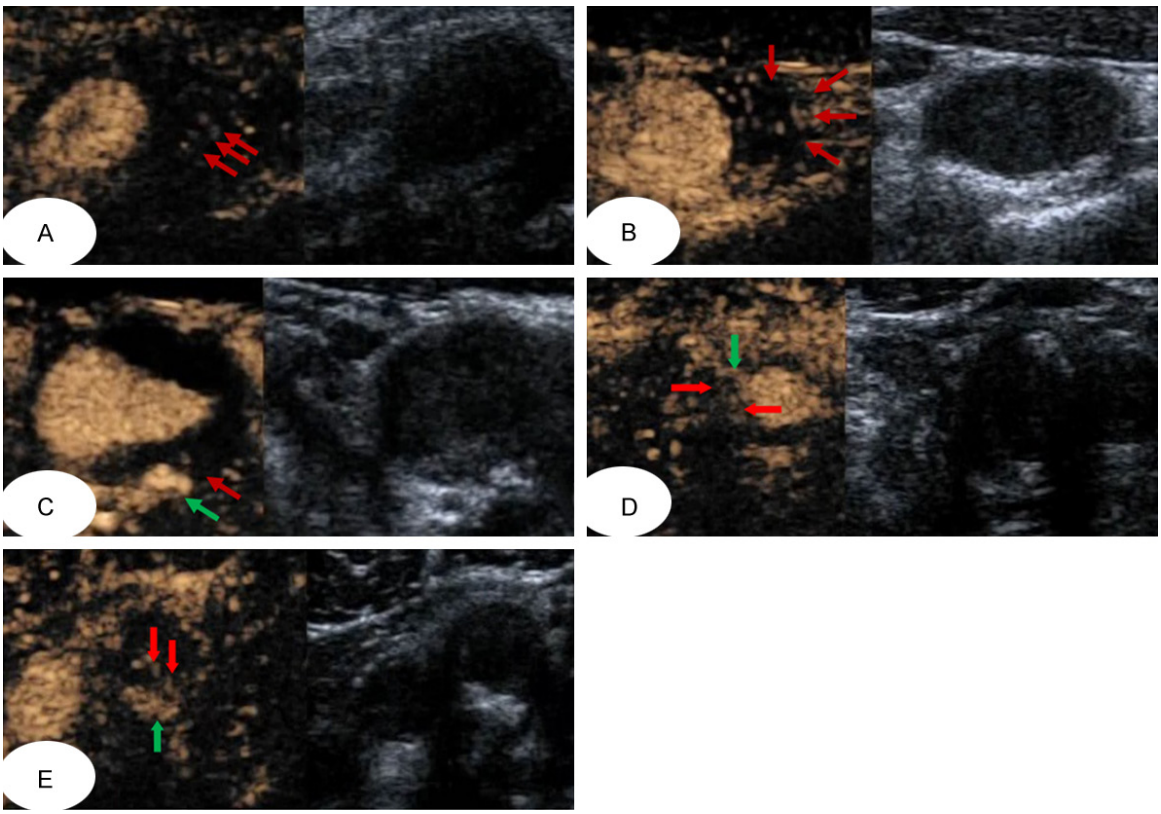

Figure 3. Representative contrast-enhanced ultrasound images showing different locations of plaque calcification. (A) Is a 60-year-old man. The red arrows point to enhancement near the base of the plaque in the bifurcation of the left carotid artery, which is grade I with a small amount of enhancement. (B) Is a 69-year-old man. The red arrows point to enhancement between the base of the plaques near the intima of the plaques, which was grade II with a large number of enhancements. (C, D) Are a 63-year-old man and a 60-year-old man respectively. The red arrow of (C) points to a small amount of enhancement with calcification peripheral, the red arrows of (D) point to a large amount of enhancement with calcification peripheral, and the green of (C and D) point to calcification near the base of the plaque. (E) Is a 60-year-old man. The red arrows of (E) point to a large amount of enhancement with calcification peripheral, and the green of (E) points to calcification near the plaque surface.

pulse inversion to optimize the depiction of the IV contrast agent and minimize echoes from the surrounding tissues. An initial bolus injection with 1.6 ml of contrast agent was quickly performed into the superficial vein in the elbow, immediately followed by 5 ml of 0.9% normal saline solution. At the same time, the built-in timer and image acquisition were started.

Table 1. Differences in the clinical characteristics of the symptomatic group and the asymptomatic group

Group	N	Men, n (%)	Hypertension, (%)	Diabetes mellitus, n (%)	Hypercholesterolemia, n (%)	Current smoker, n (%)
Symptomatic group	38	36 (94.7)	27 (71.1)	9 (23.7)	4 (10.5)	23 (60.5)
Asymptomatic group	31	25 (80.6)	16 (51.6)	2 (6.5)	7 (22.6)	16 (51.6)
χ^2		1.656	1.380	2.271	1.386	0.310
P		0.307*	0.240	0.197*	0.378*	0.578

*Fisher's exact test was used because 2 cells (50.0%) have an expected count less than 5.

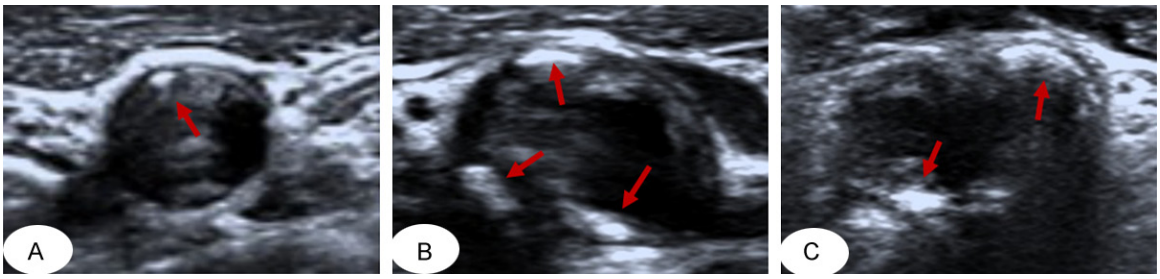

Figure 4. Representative two-dimensional conventional ultrasound images showing calcifications of plaque. A. Shows the calcified nodules of the plaque surface. B. Shows the multiple calcifications with nodules of the surface and strip calcifications of the basal in plaque. C. Is calcified nodules and strip calcifications of the plaque basal.

Table 2. The comparison with different calcification positions in plaque between the symptomatic group and the asymptomatic group determined by two-dimensional ultrasound characteristics

Group	N	Only near plaque basement	Only near plaque surface	Calcification at the surface and basal part of plaque
Symptomatic group	38	16	7	15
Asymptomatic group	31	17	4	10
χ^2			1.150	
P			0.563	

Table 3. Comparison of different calcification morphologies in plaque between the symptomatic group and the asymptomatic group as determined by two-dimensional ultrasound characteristics

Group	N	Nodular calcification	Strip calcification	Mixed morphologic calcification
Symptomatic group	38	21	8	9
Asymptomatic group	31	9	17	5
χ^2			8.561	
P			0.014	

During the examination, the probe was maintained to clearly display the sections of interest. Supplementing 1.0 ml of contrast agent successively ensures better observation. After continuous observation for 5 min, and automatic sequence ac-quisition, the dynamic images were stored in the hard disk. After examination, the images were stored in a raw data format, followed by an in-machine and off-machine quantitative analysis.

Following the infusion of the ultrasound contrast agent, the lumen of the carotid artery was enhanced, resulting in visualization of enhanced plaque luminal morphology. The presence of blood flow "activity" was identified on the basis of the dynamic movement of the echogenic reflectors (microspheres) in the intraplaque micro vessels. In the all process of contrast enhanced ultraso und, we observed the distribution characteristics of the contrast agent microbubbles perpendicular to the thickness of the plaques. According to the following criteria in this study, Grade 0 in CEUS: no enhancement in plaque. Grade I in CEUS: the only enhancement appeared between the

Table 4. The comparison with the calcification number in plaque between the symptomatic group and the asymptomatic group determined by two-dimensional ultrasound characteristics

Group	N	Calcification	
		Solitary	Multiple
Symptomatic group	38	12	26
Asymptomatic group	31	19	12
χ^2		6.091	
P		0.014	

Table 5. The comparison of enhancement characteristics in carotid plaque between the symptomatic group and the asymptomatic group by CEUS

Group	N	CEUS	
		Grade I	Grade II
Symptomatic group	38	10	28
Asymptomatic group	31	18	13
χ^2		7.137	
P		0.008	

Table 6. The comparison of calcification peripheral enhancement in carotid plaque between the symptomatic group and the asymptomatic group by CEUS

Group	N	Calcification peripheral enhancement by CEUS	
		Yes	No
Symptomatic group	38	18	20
Asymptomatic group	31	4	27
χ^2		9.338	
P		0.002	

Table 7. Comparison of the results of carotid plaques by H&E staining between the symptomatic group and the asymptomatic group

Group	N	Fibrous cap fracture	Intra-plaque hemorrhage
Symptomatic group	38	25	23
Asymptomatic group	31	5	7
χ^2		17.133	10.003
P		< 0.001	0.003

middle of the plaques and the base of the plaques, which was Grade I with a small amount of enhancement (**Figure 3A**). Grade II in CEUS: the enhancement appeared at the center of plaque and the surface of the near patch., which was Grade II with a large number of enhancements (**Figure 3B**). At the same time, it was observed whether the calcification peripheral was enhanced (**Figure 3C**).

Pathological examination

A total of 69 patients underwent carotid endarterectomy, and 69 carotid plaques were removed. The samples were fixed with 4% formaldehyde and cut into 4-um slices. The sections were stained with H&E to observe the plaque fibrous cap and hemorrhage. The sections were subjected to immunochemical staining to examine the level of CD31, a transmembrane glycoprotein that is mainly expressed by endothelial cells. The level of CD31 was used to indicate the density of neovascularization in the plaque.

Database and statistical analysis

Great care was taken to ensure an adequate blinding of all the investigators. In particular, the histological examination of the CEA specimens and the ultrasound examination of the carotid plaque was performed entirely independently. All data were analyzed with SPSS 17.0 (IBM SPSS., Chicago, IL, USA). The results are expressed as the mean ± standard deviation (SD). Measurement data were compared using Student's *t* test. Categorical variables were compared using a chi-square test. *P* values less than 0.05 were considered statistically significant.

Results

Patient characteristics

Sixty-nine patients with satisfactory image quality were analyzed. The differences in the clinical characteristics are reported in **Table 1**. The average age of the symptomatic group was similar to that of asymptomatic group (61.90 ± 6.46 vs. 62.34 ± 6.20 years, t = -1.693, P = 0.091). There was no difference in sex composition, hypertension, diabetes, hyperlipidemia, or smoking between the symptoms group and the asymptomatic group (all $P > 0.05$).

Comparison of ultrasonographic results

In this study, all subjects with satisfactory image quality and the pathology slice were analyzed and all 69 plaques were calcification of heterogeneous plaques (**Figure 4A-C**). In the

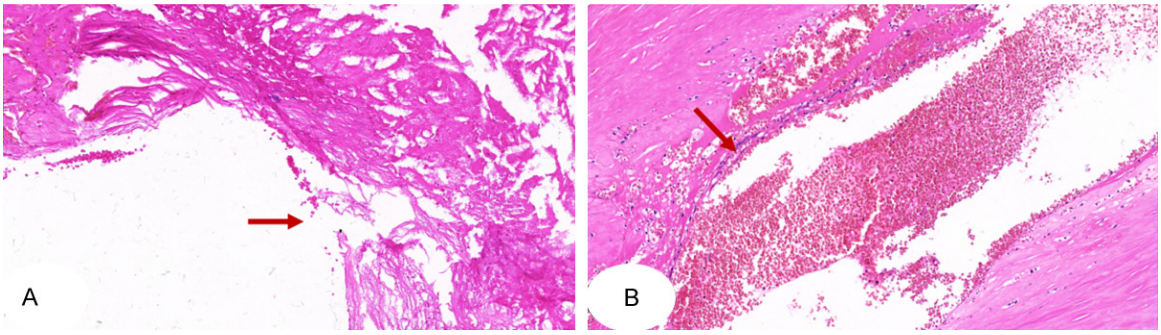

Figure 5. Representative images of H & E-stained plaque sections. The arrows of (A and B) point to fibrous cap fractures and intra-plaque hemorrhage by H&E staining (100×).

Table 8. Neovascularization density by CD31 between the symptomatic group and the asymptomatic group determined by immunohistochemical staining

Group	N	Neovascularization density (mm^2)
Symptomatic group	38	16.74 ± 4.34
Asymptomatic group	31	7.67 ± 2.60
t		7.584
P		0.000

two groups, there was no significant difference in terms of the calcification location (**Table 2**, P > 0.05 respectively). However, there were significant differences in the nodular calcification and strip calcification between the symptomatic group and the asymptomatic group in **Table 3**, which showed that the nodular calcification in the symptomatic group was more than it was in asymptomatic group (P < 0.05), and, on the contrary, the strip calcification in the asymptomatic group was more than it was in the symptomatic group (P < 0.05). There was no significant difference in the mixed morphologic calcification between the two groups (P > 0.05). It was a remarkable difference with the calcification quantity between the symptomatic group and the asymptomatic group (see **Table 4**, P < 0.05). The calcification number in the symptomatic group was more than it was in the asymptomatic group.

In this study, there was no Grade 0 in CEUS in the plaque. We saw differences with Grade I and Grade II in CEUS between the symptomatic group and the asymptomatic group with an enhancement characteristic in the carotid plaque by CEUS (χ^2 = 7.137, *P* = 0.008) (**Table 5**). The detection of Grade II in CEUS occurred more in the symptomatic group than it did in the asymptomatic group, and the detection of Grade I in CEUS occurred more in the asymptomatic group than it did in the symptomatic group.

There were differences with calcified peripheral enhancement between the symptomatic and asymptomatic groups, with the characteristics in the carotid plaque by CEUS (χ^2 = 9.338, *P* = 0.002) (**Table 6**). The calcified peripheral enhancement was higher in the symptomatic group than it was in the asymptomatic group.

Comparison of pathology results

There was a statistically significant difference with the fibrous cap fracture and the intraplaque hemorrhage in the H&E staining between the symptomatic and asymptomatic groups (χ^2 = 17.133, *P* < 0.001, χ^2 = 10.003, *P* = 0.003) (**Table 7**; **Figure 5A**, **5B**). The incidence of fibrous cap fracture and hemorrhage in the carotid plaque in the symptomatic group was higher than it was in the asymptomatic group. There was a remarkable difference in CD31 between the symptomatic group and the asymptomatic group (*t* = 7.584, *P* = 0.000) (**Table 8**; **Figure 6A-F**). The neovascularization density in the symptomatic group was higher than it was in the asymptomatic group.

Discussion

Carotid atherosclerotic disease is one of the main causes of cerebral stroke. The stability of carotid plaques is closely related to the occurrence of cerebrovascular diseases. About 23% of ischemic strokes are caused by carotid ath-

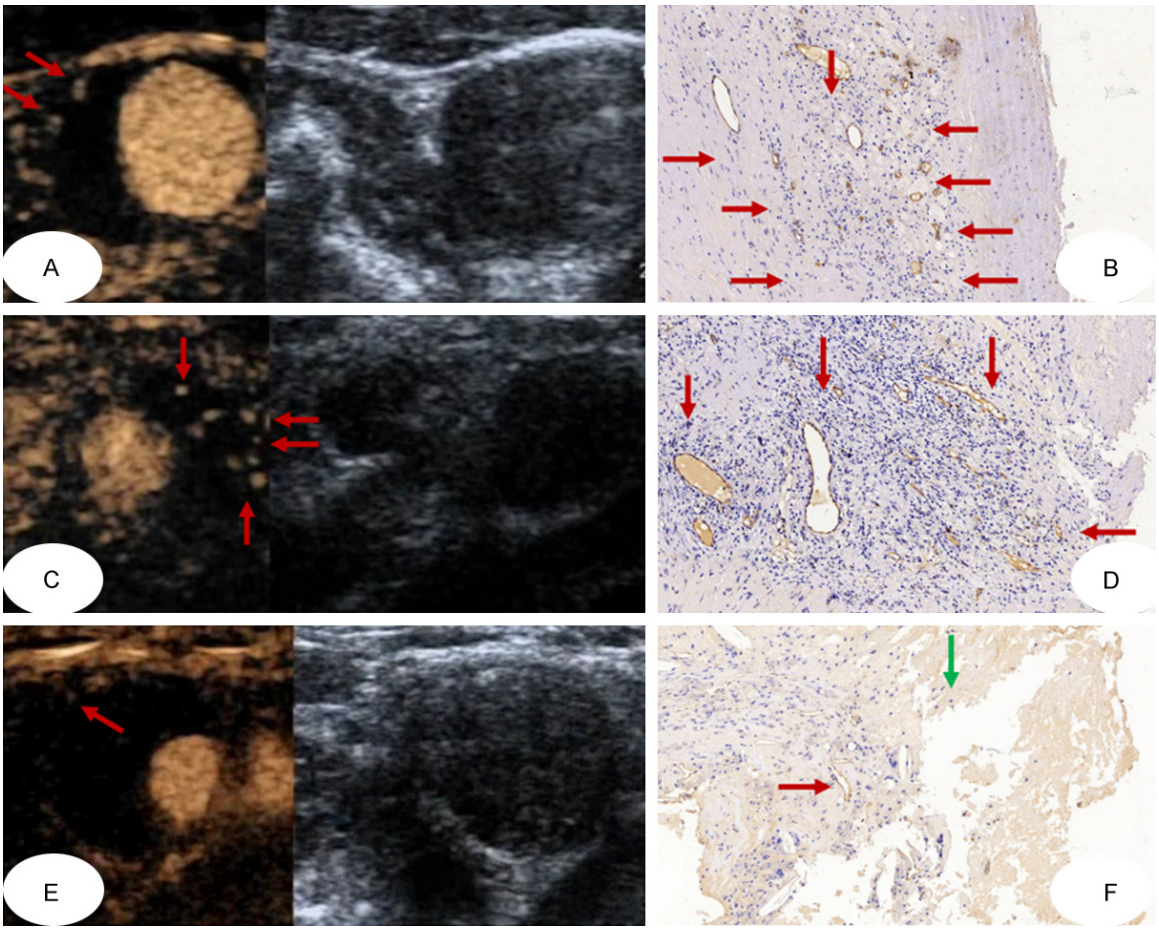

Figure 6. Representative images of ultrasonographic images and immunochemically stained plaque sections. (A and B) Are images of a 62-year-old man. The red arrows of A point to enhancement near the base of the plaque in the bifurcation of the right carotid artery, which is grade I with a small amount of enhancement. (B) Is an immunohistochemical image showing CD31 expression. (C and D) Are images of a 60-year-old man. The red arrows of C point to enhancement of the plaque in the bifurcation of the left carotid artery, which is grade II with a large amount of enhancement. (D) Is an immunohistochemical image showing CD31 expression. (E and F) Are images of a 75-year-old woman. The red arrow of (E) is calcification peripheral enhancement, and (F) is an immunohistochemical image showing CD31 expression. The red arrow of (F) marks neovascularization, the green arrow of (F) indicates calcification (B, D, F, 100×).

erosclerosis [8], and at least 20% of ischemic strokes are thromboembolic in nature [9], caused by a thromboembolism from an atherosclerotic plaque at the carotid bifurcation or the internal carotid artery. Therefore, stroke remains a massive public health problem, and there is an indispensable need for better strategies for the prevention and treatment of this disease. If the risk factors of pre-cerebral apoplexy can be found, the occurrence of stroke and cerebral infarction could be prevented. Previous studies of carotid atherosclerosis plaques have emphasized the stability of hypoechoic and hyperechoic plaques. Much of the research [10-13] on carotid artery plaques in recent years has examined how closely the formation and development of vulnerable plaque is related to neovascularization. Hemorrhage and rupture of these plaques occurs more easily, resulting in a series of clinical symptoms. Current studies have confirmed that [14, 15] a relationship between the formation of neovascularization in the carotid atherosclerotic plaque and cardiovascular events caused by the plaque falling off.

In this study, the location, shape, and quantity of calcification in the plaque were observed by two-dimensional ultrasound, and the differences between the symptomatic group and the

asymptomatic group were observed, and the results showed that there were no significant relationships between the calcification position and the clinical symptoms, but the calcification shape and quantity are related to the clinical symptoms. The nodular calcification in the symptomatic group was more than it was in the asymptomatic group. This indicated that nodular calcification is more likely to lead to plaque rupture and plaque hemorrhage, which was also demonstrated by the H&E staining in this study, which is in line with Teng et al. and Meina et al. [16, 17]. It is now thought that calcium nodules are one of the morphological features of vulnerable plaque [18, 19]. This may be due to the fact that nodular calcification is close to a round or oval shape, so the surface area of nodular calcification is larger. When the patch is partially exposed to external stress, nodular calcification is more likely to do great damage to its peripheral components, such as leading to a neovascularization rupture and hemorrhage in the plaque. On the contrary, the strip calcification in the symptom group was less than it was in the asymptomatic group. This suggests that the strip calcification in plaque is more stable.

Multiple calcification in the symptomatic group was greater than it was in the asymptomatic group, indicating that a large number of calcifications can lead to plaque instability, which agrees with Kwee's study finding that symptomatic plaques have less calcification than asymptomatic plaques [20]. The reason may be that multiple calcification in plaque, especially calcification focused on a concentrated area in the plaque being more likely to cause plaque damage and bleeding [21] and affecting the stability of the plaque [22] and increasing the risk of plaque rupture.

Neovascularization in vulnerable plaques is currently considered the most potent, independent predictive factor of plaque rupture [23], and which can promote the development of atherosclerotic lesions and even induce intraplaque hemorrhage, plaque rupture, and complications. Enhanced intensity through CEUS was closely related to clinical symptoms. A higher density of neovascularization is associated with an increased risk of typical clinical representations [24]. The results of this study showed that the enhancement of the plaque center and the enhancement of the near surface were more common in the symptomatic group and scarcer in the asymptomatic group. However, the enhancement of the basal part of the asymptomatic group was more common. The CD31 new blood vessel density of our research also proves this point, that is, the density of neovascularization in the plaque of the symptomatic group is significantly higher than it is in the asymptomatic group. Meanwhile, the incidence of neovascularization around the peripheral calcification was higher in the symptomatic group, indicating that the stability of the plaque was worse. It may be that calcification increases the rupture risk of the neovasculature, leading to intraplaque bleeding, secondary thrombosis, and ultimately stroke.

In summary, we found that the morphology and quantity of calcification and neovascularization around the peripheral calcification could be closely related to stroke in our research. Nodular calcification, multiple calcification, and neovascularization around the peripheral calcification may be risk factors for stroke, which can predict the occurrence of stroke and provide an objective basis for earlier clinical prevention.

Disclosure of conflict of interest

None.

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