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Original Article

Profile of genetic variations in severely calcified carotid plaques by whole-exome sequencing

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ABSTRACT

Background: The precise mechanisms of carotid calcification and its clinical significance have not been established.

Methods: We classified ten plaques from carotid endarterectomy patients into high- and low-calcified plaques based on the Agatston calcium scores. We performed whole-exome sequencing for genetic profiles with single nucleotide variations (SNVs), insertions, and deletions. Bioinformatic data mining was then conducted to disclose specific gene variations to either high- or low-calcified carotid plaques.

Results: In the carotid plaques, G:C>A:T/C:G>T:A transitions as SNVs, insT after C/insC after A as insertions, and delA after G/delT after C as deletions were most frequently observed, but no significant difference was observed between the high- and low-calcified plaque groups in their proportion of base-pair substitution types. In the bioinformatic analysis, SNVs of ATP binding cassette subfamily C member 6 (*ADCC6*) were more commonly found in high-calcified plaques and SNVs of *KLKB1* were more commonly found in low-calcified plaques compared to the other group. No new genetic variants related to calcification or atherosclerosis among those not registered in dbSNP was detected.

Conclusion: Our findings clarified the features of base-pair substitutions in carotid plaques, showing no relation to calcification. However, genetic variants in *ADCC6* relating to vascular calcification for high-calcified plaques, and in *KLKB1* encoding kallikrein associated with vascular regulation of atherosclerosis for low-calcified plaques were more specifically extracted. These results contribute to a better understanding of the genetic basis of molecular activity and calcium formation in carotid plaques.

Keywords: Calcification, Carotid endarterectomy, Carotid plaque, Single nucleotide variation, Whole exome sequencing

INTRODUCTION

Calcification in carotid plaques is often encountered in patients with asymptomatic carotid stenosis, and this calcification has been thought to be stable and hardly evoke symptoms compared to so-called "soft" vulnerable plaques.[11,15,21] However, many investigators have reported an association between calcification in coronary plaques and clinical symptoms that are compatible with an acute coronary syndrome or cardiovascular events.[15,27,28] Although the reason(s) for this difference and the clinical significance of vascular calcification (as well as the precise mechanism underlying its formation) have been discussed,[5,11,29] they have not been fully elucidated.

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Single nucleotide polymorphisms (SNPs) are naturally occurring variants that affect a single nucleotide and are present in >1% of a population. SNPs are present in the germline of an individual, whereas single nucleotide variations (SNVs) are single-base differences among individuals, and their percentage in a population is not a factor; SNVs are also observed in somatic cells. Although atherosclerotic diseases such as cerebrovascular disease (CVD) and coronary artery disease (CAD) are multifactorial disorders, and information about the genetic associations in their pathogeneses is limited, associated SNPs have been reported in trials seeking influential rare variants or genetic biomarkers[33] by genome-wide association studies (GWASs) of peripheral blood mononuclear cells.[31] In the GWASs conducted to date, however, it was quite difficult to obtain reproducible results (especially for stroke) even with cohorts of ~1000 subjects. Recently, a meta-analysis of 67,162 patients with CVDs and 454,450 controls by the MEGASTROKE consortium revealed 32 susceptibility loci and associated genes, including *FGA, PDA3A,* and *PLG*.[19]

In the present study, we used an approach that differed from those used in the above-cited conventional studies of CVD that sought to identify SNPs using large cohorts and peripheral blood sampling. We attempted to determine the differences in variations of genes by whole-exome sequencing (WES), according to the presence and amount of calcification in carotid plaque specimens that were obtained during carotid endarterectomy (CEA) for patients with carotid stenosis. We applied bioinformatic data mining to disclose the specific susceptibility gene variations associated with high- or low-calcified plaques to gain a better understanding of the genetic basis for calcification in carotid plaques.

MATERIALS AND METHODS

Patients and specimens

Carotid plaques from ten endarterectomy patients were investigated [Table 1]. Half of the plaques were highly calcified plaques (H1–5) that showed a mean calcium score of 2315.8 ± 389.4 and the other five plaques were low-calcified (L1–5) with a mean calcium score of 22.1 ± 33.5. We evaluated the characteristics of the plaques other than calcification (i.e., lipid core, fibrous tissue, and hemorrhage) by examining hematoxylin/eosin-stained specimens, and we stratified each finding into three degrees or none. The degrees of stenosis were higher in the high-calcified plaques compared to the low-calcified plaques (81.2 ± 7.2% vs. 73.4 ± 14.6%, $P < 0.01$). No remarkable difference was found between the high- and low-calcified plaques concerning other clinical data except for the patients' rates of hypertension and diabetes [Table 1].

All of the patients enrolled in this study received a detailed explanation of the nature of the project, and all gave written

Table 1: The characteristics of the plaques and the patients' clinical data.

Plaque	Ca score	Lipid core	Fibrous tissue	Hemorrhage	Age	Gender	Stenosis (%)	Symptom	Hypertension	Diabetes mellitus	Dyslipidemia	IHD w/PCI	Renal malfunction	Smoking	Antiplatelet	Anti-coagulant
H1	1490.7	–	+	–	77	F	75.0	S	+	+	+	+	–	–	+	–
H2	1789.0	+	+	–	60	M	82.0	A	+	+	–	–	–	+	+	–
H3	2262.9	+	+	–	77	F	90.0	S	+	–	+	–	–	–	+	–
H4	2934.5	+	+	–	72	M	73.0	S	+	–	+	–	–	+	+	–
H5	3102.1	–	+	–	67	M	86.0	A	+	–	+	–	–	+	+	–
L1	0	+	++	–	69	M	74.0	S	+	–	–	–	–	–	+	–
L2	0	+	+	–	76	M	60.0	S	+	–	+	–	–	+	+	–
L3	0	+	++	–	52	M	95.0	S	–	–	–	–	–	–	+	–
L4	34.7	++	++	+	69	M	60.0	S	–	–	+	–	–	+	+	–
L5	75.6	+++	+	–	63	M	78.0	S	–	–	–	–	–	+	+	–

H1-5: High-calcified plaques, L1-5: Low-calcified plaques, M: Male, F: Female, A: Asymptomatic, S: Symptomatic, IHD: Ischemic heart disease, PCI: Percutaneous coronary intervention

informed consent before undergoing the CEA. Approval from the local ethical committee for human genetic research was also obtained.

The patients underwent preoperative multi-detector computed tomography angiography (MDCTA) that identified the plaque location and the degree of stenosis. The MDCTA was performed with a 64-detector row CT scanner (SOMATOM Definition: Siemens Medical Solutions, Forchheim, Germany). An evaluation of the calcification of the plaques using the Agatston calcium score was also performed with non-contrast-enhanced CT obtained before the contrast media injection for MDCTA, as described elsewhere.[11-13]

Briefly, the calcification of each carotid plaque was quantified using specialized software run on a workstation (Ziostation ver. 1.17; Amin, Tokyo) with the preoperative MDCTA data. The Agatston calcium score was calculated as the product of the areas of calcified lesions and the weighted signal intensity scalars, dependent on the maximal Hounsfield unit (HU) value within the lesion (scalar = 1 if 130–199 HU, 2 if 200–299, 3 if 300–399, and 4 if ≥400).

All carotid plaques were obtained during a CEA, and specimens containing portions consisting of the most severe stenosis were harvested. This enabled the sampling of tissues with high calcification for the plaques with high calcium scores and the sampling of tissues with less calcification for the plaques with low calcium scores. All samples were stored at −80°C and thawed only once.

WES

Genomic DNA was extracted from fresh frozen carotid plaques using a Nucleospin Tissue Kit (Macherey Nagel, Düren, Germany) following the manufacturer's instructions. DNA was qualified using a NanoDrop and agarose gel electrophoresis. DNA libraries were prepared and exome enrichment was performed by SureSelect XT Human All Exon V6 (Agilent Technologies, Santa Clara, CA). The 100-bp end sequencing of exomes was carried out on the HiSeq2500 genome analyzer platform (Illumina, San Diego, CA). The sequencing raw reads were mapped to the UCSC hg19/GRCh37 reference human genome by the Burrows-Wheeler Aligner-MEM ver. 0.7.15. Duplicate marking and base quality recalibration were performed using Picard ver. 2.2.4 (https://broadinstitute.github.io/picard) and genome analysis toolkit (GATK) ver. 3.6 (Broad Institute, Cambridge, MA) for strict quality control. SNVs and small insertions and deletions (indels) were called by GATK.

Bioinformatics data mining

All detected variants were filtered to exclude low-quality variants with the use of GATK by choosing those with "PASS" in the FILTER field. The effects of alterations of amino acids and frameshift variants on the protein structure and function were predicted as the Annotation Impact of "HIGH" or 'MODERATE' by SnpEff ver. 4.2.[3] For the estimation of the effects on missense mutations in dbNSFP ver. 3.0c (https://sites.google.com/site/jpopgen/dbNSFP), the Functional Analysis Through Hidden Markov Models score ≤−1.5 (meaning "DAMAGING") was applied.[32] The effects on clinical disorders were evaluated with ClinVar (file date 20170905, https://www.ncbi.nlm.nih.gov/clinvar), annotated as "pathogenic."

Among the gene variations that satisfied all of the above-noted conditions, we compared the high- and low-calcified plaque groups and chose the variations with the mean allele frequency (AF) and a fold change >2 compared to the other group. We then compared all 25 individual sets of the two groups (one of five high-calcified plaques vs. one of five low-calcified plaques) and selected the unique gene variations detected in only the high- or low-calcified plaque group in >30% of the comparison sets. In both comparisons, we excluded the gene variations with 0.4≤ (mean) AF ≤0.6 that were suspected to have higher possibilities of germline origin. The gene variations that were detected in both groups and in individual comparisons were extracted as the final results.

To identify unknown novel gene variations in the calcified carotid plaques, we selected genes that were not included in the database SNPs (dbSNP) (http://www.ncbi.nlm.nih.gov/snp) after choosing gene variations with "PASS" on the FILTER of GATK and "HIGH" and "MODERATE" on the Annotation Impact of SnpEff. We then extracted gene variations unique to either the high- or low-calcified plaques, using gene variations with a mean AF >0.6 by the group comparison. The variations with a mean AF of fold change >2 compared to the other group were selected as unique gene variations. We also compared the variations between individual specimens (five high- vs. five low-calcified plaques) and selected the variations with an AF >0.6, following filtering with "PASS" on the FILTER of GATK and "HIGH" on the Annotation Impact of SnpEff.

We performed an enrichment analysis for biological pathways of genes using the functional annotation clustering algorithm on the database for annotation, visualization, and integrated discovery (DAVID ver. 6.8, https://david.ncifcrf.gov/), which is a web-based integrated biological knowledge base and annotation tool. The analysis included the gene ontology biological process, cellular components, and molecular functions. Molecular interactions and reaction and relation networks were also studied with the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/kegg/kegg_ja.html).

Statistical analyses

All statistical evaluations were performed with the statistical software SPSS ver. 22.0 (SAS, Cary, NC, USA) and StatMate

III (ATMS, Tokyo), and all results are presented as mean ± SD values. The Mann–Whitney U-test and the Chi-square test with Yates' correction were used to compare the demographic data between the patients with high- and low-calcified plaques. The between-group differences in the proportions of variables were evaluated with Spearman's rank correlation coefficient, and those between individual specimens were evaluated with Pearson's correlation coefficient. $P < 0.05$ was considered significant.

RESULTS

Genetic variants detected by the WES

About 99.6–99.7% of the sequence reads were mapped to the UCSC hg19 reference human genome. The coverage rate was 83.1–91.2% by ≥20 unique reads, and 72.4–74.0% of the target regions were covered. Of all 13,783,666 detected variants, SNVs, insertions, and deletions accounted for 88.0%, 5.3%, and 6.8%, respectively. The proportions of the identified principal variants in the targeted exome region are shown in [Figure 1]. Synonymous and missense variants represented the most common types of variants in all specimens (ñ = 1, $P < 0.01$). Similar proportions of variants were observed in the high- and low-calcified plaque groups.

The percentages of SNVs (r = 0.94–0.99, $P < 0.001$), insertions (r = 0.55–0.93, $P < 0.001$), and deletions (r = 0.72–0.96, $P < 0.01$ or $P < 0.001$) in individual plaques shown in [Figure 2a-c] demonstrated no remarkable difference in each specimen for base-pair substitution.

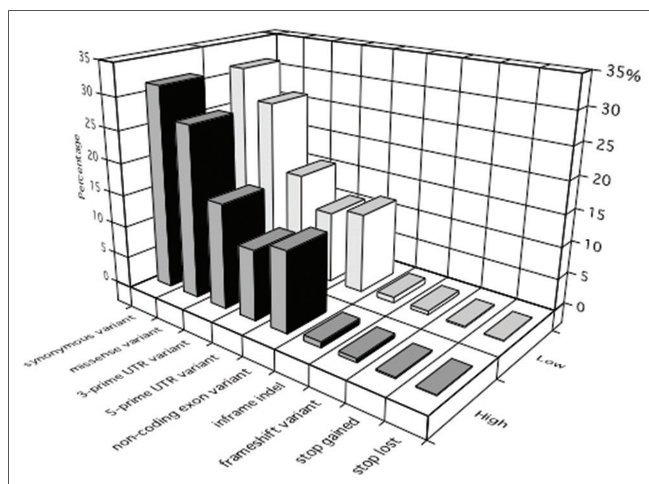

Figure 1: The proportions of the identified principal variants in the targeted exome region. Synonymous and missense variants represented the most common types of variants in all specimens. Similar proportions of variants were observed in the high- and low-calcified plaque groups. High: high-calcified plaques, Low: low-calcified plaques.

Regarding the SNVs, G:C>A:T and C:G>T:A transitions were the most frequent followed by T:A>A:T transversion and A:T>G:C transition [ρ = 1, $P < 0.01$, Figure 3a]. The most frequent insertions were insT after C and insC after A, followed by insA after T, insT after A, insG after A, and insT after G [ρ = 1, $P < 0.01$, Figure 3b]. Among the deletions, delA after G and delT after C were the most common followed by delA after T and delA after C [ρ = 0.99, $P < 0.01$, Figure 3c]. No significant differences in nucleotide changes between the high- and low-calcified plaque groups were apparent for any of the SNVs and indels, except for a slight difference in A:T>G:C transversion [Figure 3a].

Specific variants for the high- and low-calcified carotid plaques in the dbSNP

After the filtering was performed under the conditions described above, ten variations were extracted [Table 2]. The high- and low-calcified plaque groups were compared, and we chose the gene variations showing a mean AF with a fold change >2 compared to the other group. In the high-calcified plaque group, one mutation of *ABCC6* (G>T transversion at 16278633 (rs8058694) was selected. In the low-calcified plaque group, *KLKB1* (G>A transition at 187158034 (rs3733402) and *SERPINA7* (C>A transversion at 105278361 (rs1804495) were extracted.

We then performed individual comparisons of high-calcified versus low-calcified specimens for 25 (5 × 5) sets. *ABCC6* was chosen in 32% of the sets (8/25) for the high-calcified group. *KLKB1* was selected in 48% of the sets (12/25) for the low-calcified group, but *SERPINA7* was extracted only in 16% (4/25) [Table 3].

Specific variants for high- and low-calcified carotid plaques outside of the dbSNP

We attempted to identify novel variations by examining those that were not present in the dbSNP database. After the filtering under the conditions noted above was conducted, 98 variations in high-calcified plaques were those with a mean AF >0.6, and among them *ANP32E, C2orfB1, GPRIN1*, and *GPX6* were variations of changes with a mean AF more than twofold that of the low-calcified group. On the other hand, 118 variations with a mean AF >0.6 were chosen in the low-calcified plaque group, and we selected *OR12D1, KATAP5-1,* and *KATAP5-2* as variations of changes with a mean AF more than twofold that of the high-calcified group [Table 4].

Our comparisons of individual samples between the high- and low-calcified plaque groups revealed five gene variations unique to high-calcified plaques and 13 variations unique to low-calcified plaques in >30% of all 25 sets [Table 5].

We conducted a trial for the functional enrichment of biological pathways for the above-noted detected genes

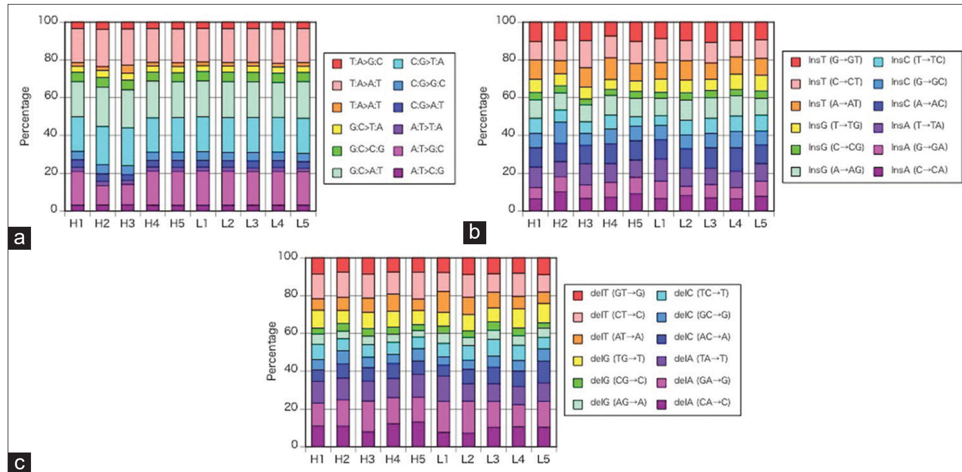

Figure 2: (a) The percentages of single nucleotide changes in individual plaques demonstrated no remarkable difference in each specimen for base-pair substitution. (b) The percentages of insertions in individual plaques demonstrated no remarkable difference in each specimen for base-pair substitution. (c) The percentages of deletions in individual plaques demonstrated no remarkable difference in each specimen for base-pair substitution. H1–5: high-calcified plaques, L1–5: low-calcified plaques.

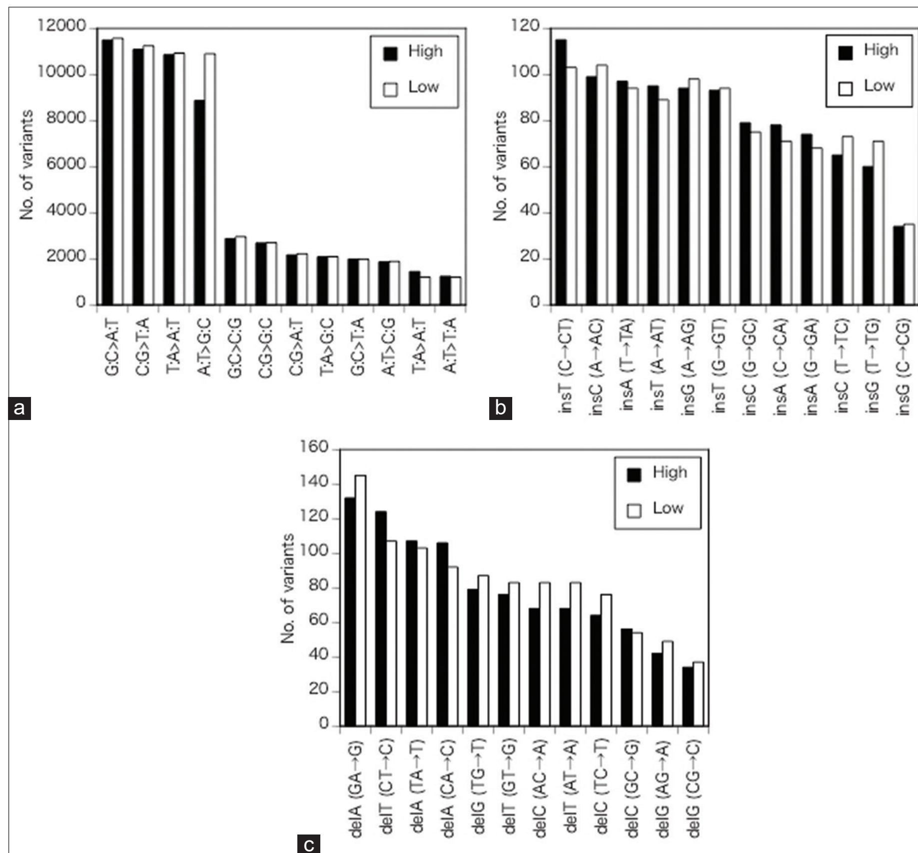

Figure 3: (a) Details of the alterations of single nucleotides. Regarding SNVs, G:C>A:T and C:G>T:A transitions were the most frequent followed by T:A>A:T transversion and A:T>G:C transition. No significant differences in nucleotide changes between the high- and low-calcified plaque groups were apparent for the SNVs, except for a slight difference in A:T>G:C transversion. (b) Details of the alterations of single nucleotides. The most frequent insertions were insT after C and insC after A, followed by insA after T, insT after A, insG after A, and insT after G. No significant differences in nucleotide changes between the high- and low-calcified plaque groups were apparent for the ins. (c) Details of the alterations of single nucleotides. Among the deletions, delA after G and delT after C were the most common followed by delA after T and delA after C. No significant differences in nucleotide changes between the high- and low-calcified plaque groups were apparent for the deals. High: high-calcified plaques, low: low-calcified plaques.

Table 2: Specific variants for high- and low-calcified carotid plaques in dbSNP by group comparison.

Chromosome	Position	Reference	ID	Annotation	Annotation impact	ClinVar	FATHMM score	Gene name	mAF (H)	mAF (L)	H/L	L/H
chr1	27238527	C>A	rs74315350	Missense	Moderate	Pathogenic	−4.1	NR0B2	0.00	0.12	0.00	-
chr3	136046026	C>T	rs121964959	Missense	Moderate	Pathogenic	-4.7	PCCB	0.00	0.09	0.00	-
chr4	187158034	G>A	rs3733402	Missense	Moderate	Pathogenic	−2.3	KLKB1	0.18	0.89*	0.21	4.87†
chr5	176520243	G>A	rs351855	Missense	Moderate	Pathogenic	−1.7	FGFR4	0.46	0.31	1.50	0.67
chr7	80286003	C>T	rs75326924	Missense	Moderate	Pathogenic	−3.3	CD36	0.00	0.08	0.00	-
chr16	16278863	G>T	rs8058694	Missense	Moderate	Pathogenic	−2.6	ABCC6	0.37*	0.17	2.15†	0.46
chr16	16281007	A>G	rs12931472	Missense	Moderate	Pathogenic	−2.6	ABCC6	0.45	0.21	2.19†	0.46
chr17	73759113	G>A	rs80084721	Missense	Moderate	Pathogenic	−2.0	GALK1	0.09	0.09	0.96	1.05
chr22	19753444	G>A	rs41298838	Missense	Moderate	Pathogenic	−2.1	TBX1	0.09	0.09	1.00	1.00
chrX	105278361	C>A	rs1804495	Missense	Moderate	Pathogenic	−2.8	SERPINA7	0.06	0.20*	0.31	3.25†

mAF: Mean allele frequency, H: High-calcified plaques, L: Low-calcified plaques. NR0B2: Nuclear receptor subfamily 0 group B member 2, PCCB: Propionyl-CoA carboxylase beta chain, KLKB1: Plasma kallikrein B1, FGFR4: Fibroblast growth factor receptor 4, CD36: Platelet glycoprotein 4, ABCC6: ATP-binding cassette, subfamily C, member 6. GALK1: galactokinase 1, TBX1: T-box transcription factor, SERPINA7: Thyroxine-binding globulin precursor. *mAF<0.4 or mAF>0.6, †Fold change>2

Table 3: Specific variants for high- and low-calcified carotid plaques in dbSNP by individual comparisons.

Chromosome	Position	Reference	ID	Annotation	Annotation impact	ClinVar	FATHMM score	Gene name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
chr3	49568275	G>A	rs117209107	ms	m	p	−5.2	DAG1																									
chr3	38645420	T>C	rs1805124	ms	m	p	−3.5	SCN5A																									
chr4	88533540	A>T	rs36094464	ms	m	p	−2.6	DSPP																									
chr4	187158034	G>A	rs3733402	ms	m	p	−2.3	KLKB1																									
chr5	176520243	G>A	rs351855	ms	m	p	−1.7	FGFR4																									
chr5	176831083	C>G	rs183643295	ms	m	p	−2.5	F12																									
chr9	116153891	C>G	rs386562149	ms	m	p	−2.1	ALAD																									
chr16	16281007	A>G	rs12931472	ms	m	p	−2.6	ABCC6																									
chr16	16251599	C>T	rs386562149	ms	m	p	−2.7	ABCC6																									
chr16	16278863	G>T	rs8058694	ms	m	p	−2.6	ABCC6																									
chrX	19375782	A>C	rs2229137	ms	m	p	−3.9	PDHA1																									
chrX	105278361	C>A	rs1804495	ms	m	p	−2.8	SERPINA7																									

ms: Missense, m: Moderate, p: Pathogenic, DAG1: Dystroglycan preproprotein, SCN5A: Sodium channel protein Type 5 subunit alpha isoform f, DSPP: Dentin sialophosphoprotein preproprotein, KLKB1: Plasma kallikrein B1, FGFR4: Fibroblast growth factor receptor 4, F12: Coagulation factor XII preproprotein, ALAD: Delta-aminolevulinic acid dehydratase isoform a, ABCC6: ATP-binding cassette subfamily C member 6, PDHA1: Pyruvate dehydrogenase E1 component subunit alpha, SERPINA7: Thyroxine-binding globulin precursor. Numbers (1-25): Each individual comparison set (high- versus low-calcified plaque). Blue and sky blue brocks: an individual comparison set in which the gene variation detected unique to the low-calcified plaques. Red and pink brocks: an individual comparison set in which the gene variation detected unique to the high-calcified plaques

Table 4: Specific variants for high- and low-calcified carotid plaques out of dbSNP by group comparison.

Calcification in plaque	Chromosome	Position	Reference	Alteration	Type	Annotation	Annotation impact	Gene name	mAF (H)	mAF (L)	H/L	L/H
High	chr1	150199039	CTCCTCT	C	DEL	Disruptive inframe deletion	Moderate	ANP32E	0.76	0.25	3.10*	0.32
	chr2	74642265	A	†††	INS	Inframe insertion	Moderate	C2orf81	0.61	0.09	7.03*	0.14
	chr5	176026121	TCAAAGACCCAGGA	T	DEL	Frameshift variant	High	GPRIN1	0.68	0.19	3.61*	0.28
	chr5	176026135	TCCTCCTTCCTC	T	DEL	Frameshift variant	High	GPRIN1	0.69	0.20	3.53*	0.28
	chr6	28483482	A	C	SNP	Missense variant	Moderate	GPX6	0.68	0.29	2.33*	0.43
Low	chr6	29385772	G	C	SNP	Stop lost	High	OR12D1	0.37	0.79	0.47	2.14*
	chr11	1606120	†	A	DEL	Disruptive inframe deletion	Moderate	KRTAP5-1	0.26	0.62	0.42	2.35*
	chr11	1619172	††	A	DEL	Disruptive inframe deletion	Moderate	KRTAP5-2	0.29	0.65	0.44	2.25*

mAF: Mean allele frequency, H: High-calcified plaques, L: Low-calcified plaques. ANP32E: Acidic leucine-rich nuclear phosphoprotein 32 family member E isoform 1, C2orf81: Uncharacterized protein C2orf81 isoform 1, outgrowth 1, GPRIN1: G protein-regulated inducer of neurite, GPX6: Glutathione peroxidase 6 precursor, OR12D1: Olfactory receptor 12D1, KRTAP5-1: Keratin-associated protein 5-1, KRTAP5-2: Keratin-associated protein 5-2. *Fold change>2. †ACCACAGCCACCCTTGGATCCCCCACAAGAG. ††ACCCCCACAGGAGCCACAGCCCCCCTTGGAG. †††ACGCGGAGGGGCGGGTGGCGCCGCC

Table 5: Specific variants for high- and low-calcified carotid plaques out of dbSNP by individual comparison.

Calcification in plaque	Chromosome	Position	Reference	Alteration	Type	Annotation	Gene name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
High	chr1	41847881	C	CGGGA	INS	fv	FOXO6																									
	chr3	195506270	*	G	DEL	sdv	MUC4																									
	chr6	31380160	TG	T	DEL	fv	MICA																									
	chr12	11506244	**	C	DEL	fv	PRB1																									
	chr12	11420391	***	T	DEL	fv	PRB3																									
Low	chr3	12046256	†	G	DEL	fv	SYN2																									
	chr3	12046268	GCGCGA	G	DEL	fv	SYN2																									
	chr3	133969487	G	GC	INS	fv	RYK																									
Low	chr5	72743299	G	GGC	INS	fv	FOXD1																									
	chr6	29911240	T	A	SNP	sg	HLA-A																									
	chr12	40880361	GCT	G	DEL	fv/sl	MUC19																									
	chr12	40880364	††	G	DEL	fv/sl	MUC19																									
	chr12	40880384	ACCATCAG	A	DEL	fv	MUC19																									
	chr12	40880286	†††	A	DEL	sl/did	MUC19																									
	chr12	40880394	GAAGTC	G	DEL	fv	MUC19																									
	chr19	43865319	CCT	C	DEL	fv/sl	CD177																									
	chr19	43865324	A	AAG	INS	fv	CD177																									
	chr19	43865324	A	AAG	INS	fv	CD177																									

fv: Frameshift variant, sdv: Splice donor variant, sg: Stop gained, sl: Stop lost, did: Disruptive inflame deletion, FOXO6: Forkhead box protein O6, MUC4: Mucin-4 isoform f precursor, MICA: MHC Class I polypeptide-related sequence A isoform 1 precursor, PRB1: Basic salivary proline-rich protein 1 isoform 2 precursor, PRB3: Basic salivary proline-rich protein 3 precursor, SYN2: Synapsin-2 isoform Iib, RYK: tyrosine-protein kinase RYK isoform 2 precursor, FOXD1: Forkhead box protein D1, HLA-A: HLA Class I histocompatibility antigen, A-1 alpha chain precursor, HLA-DQB1: HLA class II histocompatibility antigen, DQ beta 1 chain isoform 2 precursor, MUC19: Mucin-19 precursor, CD177: CD177 antigen precursor, KIR2DS4: Killer cell immunoglobulin-like receptor 2DS4 isoform 1 precursor, Numbers (1-25):each individual comparison set (high- versus low-calcified plaque). Gray brock: an individual comparison set in which the gene variation detected. *GACCTGTGGATAATGAGGAAGCATTGGTGACAGGAAGAGGGGTGGTGTC. **CGGGTGGTCCTTGTGGCTTTCCTGGAGGTGGGGGACCTTGAGGTTTGTTGCCTCCTTGTGGGGGTGGTCCTTGTGGCTTTCCTGGAGGAGGTGGGGGACCTTGGGGC TGGTTGCCTCCTTGTGGGGGTGGTCCTTGTGGCTTTCCTGGAGGAGATTGGGAACTTCGGGACTTGTCTCCTTGTGG. ***TGGACGAGGTGGGGGACCTTGGGACTGGTT TCCTCCTTGTGGGGGTGGTCCTTCTGGCTTTCCTGGACGAGGTGGGGGACCTTGAGGTTTGTTGCCTCCTTGTGGGGGTGGTCCTTCTGGCTTTCCC. †GCCCCCCCCC. ††GAAGAGACAGGGGCAAC. †††ACAACTGGACCATCAGCTGAAGAGACAGGGG. ††††TCCCGGAGCTCCTATGACATGTA

using DAVID's Functional Annotation Clustering. Regarding the seven genes detected by our group comparison, no enrichment was observed for the high- or low-calcified plaque group. [Table 6] presents the respective enriched annotations for genes unique to the high- and low-calcified plaques detected by our comparison of individual specimens; the annotations are ordered by their significance level. "Signal," "Glycoprotein," and "Glycosylation site: N-linked" were commonly enriched in both groups. Terms concerning the "Plasma membrane" were more enriched in the low-calcified plaque group. However, the enrichment scores of both annotation clusters were not high enough to be significant.

DISCUSSION

The results of our present analyses demonstrated that in carotid plaques, G:C>A:T/C:G>T:A transitions as SNVs, insT after C/insC after A as insertions, and delA after G/delT after C as deletions were most frequently observed, but no significant difference was detected between the high- and low-calcified plaque groups in their proportion of base-pair substitution types. Compared to the other group, variants of ATP binding cassette subfamily C member 6 (*ADCC6*) were more commonly found in the high-calcified plaques and variants of *KLKB1* were more commonly found in the low-calcified plaques. No new gene variants related to calcification or atherosclerosis was detected among those that were not registered in the dbSNP.

Types of base-pair substitutions in carotid plaques and the relation to calcification

To date, no specific and frequent substitutional types of SNPs or indels have been reported in the carotid artery, and little similar information is available for other aspects of atherosclerosis. In tumor studies, C:G>T:A transitions were the most frequent in parathyroid adenoma and non-functioning pituitary adenoma.[22,23] Fang *et al.*[6] reported that T:A>C:G was observed in nearly one-half of their subjects with papillary thyroid cancer (PTC), whereas the C>T/G>A substitution was the most common pattern in somatic mutations as shown by WES results for PTC.[25]

Diversity is often observed among the cases in these tumor studies, whereas in the present study, we observed a relatively homogeneous distribution of base-pair substitution types among carotid plaque specimens. This might be attributable to the difference in heterogeneity between tumor and atherosclerosis specimens. It is conceivable that SNPs of G:C>A:T and C:G>T:A were the most frequent in our present investigation because it is generally easy for a transition in which purine/pyrimidine bases turn into the same bases to occur compared to a transversion that is a substitution

Table 6: Enriched annotations for genes* unique to high- and low-calcified plaques by individual comparison.

Calcification in plaque	Category	Term	Count	*P*-value	Enrichment score
HIGH	UP_KEYWORDS	Signal	4	0.03	0.97
	UP_KEYWORDS	Glycoprotein	4	0.03	
	UP_KEYWORDS	Secreted	3	0.04	
	UP_SEQ_FEATURE	Signal peptide	3	0.07	
	UP_SEQ_FEATURE	Glycosylation site: N-linked	3	0.11	
	UP_KEYWORDS	Disulfide bond	3	0.13	
LOW	UP_SEQ_FEATURE	Signal peptide	5	<0.01	0.81
	UP_KEYWORDS	Signal	5	0.02	
	GOTERM_CC_DIRECT	GO: 0005886~Plasma membrane	5	0.03	
	GOTERM_CC_DIRECT	GO: 0005887~integral component of plasma membrane	3	0.07	
	UP_SEQ_FEATURE	Glycosylation site: N-linked	4	0.11	
	UP_KEYWORDS	Glycoprotein	4	0.13	

*Genes are shown in Table 5

between purine and pyrimidine bases. It is noteworthy that the proportions of the types of base-pair substitutions for SNPs as well as indels were mostly similar irrespective of the amount of calcification, except for the slight difference in A:T>G:C transition.

Previous genetic variant studies for atherosclerosis

Many genetic association investigations have attempted to determine the role(s) of certain gene mutations (especially SNPs) for atherosclerosis by performing GWAS or WES in large populations and meta-analyses, but the results conflict. Concerning GWASs for carotid atherosclerosis, the associated candidate genes were *MMP-3* and other MMPs, *CDKN2A/2B*, *IL-6/10*, *APOE*, *ACE*, *PON-1*, and others.[7] The variant in *PNPT1* gene (rs62165235) was associated with the quantitative carotid intima-media thickness (cIMT) in a family-based study population.[34] A meta-analysis of GWASs of a total of 71,128 individuals for cIMT and 48,434 individuals for carotid plaque traits revealed novel susceptibility loci for the cIMT: *PINX1, ADAMTS9,* and *LOXL4*.[8] The SNPs of *CDKN2A/2B* and *PHACTR1* are associated with coronary artery calcification, and they also increase the risk of the carotid artery and aortic arch calcification.[36] A recent report stated that the *ADAMTS7* variants rs3825807 and rs7173743 were related to the risk of carotid plaque vulnerability.[17]

Most of these previous studies sought to identify hereditary gene variations such as SNPs derived from the germline by investigating samples from the peripheral blood of patients. Several SNPs identified by exhaustive searches and a GWAS or WES were used to confirm the association in other conditions, diseases, or cohorts. In the present study, we extracted the whole exome from affected carotid plaque specimens and compared the genome variations in high- and low-calcified carotid plaques based on calcium scores to detect the variations that were specific to each group. We excluded gene variations with 0.4≤ AF≤0.6, which may be highly likely to have a germline derivation from the ideal AF 0.5, to narrow the obtained data to somatic acquired information as much as possible.

The relationship between detected gene variations and calcification/atherosclerosis in carotid plaques

We extracted specific gene variations for each of the two calcium score-based classified plaque groups. Variations of *ADCC6* gene, also known as multidrug resistance-associated protein 6, were more frequently detected in the present high-calcified plaque group; *ADCC6* stimulates the release of adenosine triphosphate (ATP). Pyrophosphate broken down from ATP functions as an inhibitor of the production of hydroxyapatite crystals and regulates calcification and mineralization.[16,26] The mutation of *ADCC6* gene has thus been reported to be associated with generalized arterial calcification of infancy (GACI) which exhibits abnormal calcification of arteries.[18,24,30] A mutation of *ABCC6* gene also causes pseudoxanthoma elasticum (PXE), which is characterized by abnormal accumulations of calcium and minerals in elastic fibers, including blood vessels, skin, eyes, and other tissues.[9,20] Although the variants detected frequently in the present high-calcified plaque group are not among the 13 previously reported mutations in GACI patients,[24] defects in *ADCC6* might be associated with the attenuation of the anti-calcification effect of this ligand.

Plasma kallikrein encoded by *KLKB1* – variations of which we observed more commonly in the low-calcified plaque group – cleaves high-molecular-weight kininogen to release bradykinin, which plays crucial roles in the vascular regulation of atherosclerosis by vasodilation and in the prevention of cell proliferation through the kinin-kallikrein system.[4] Plasma kallikrein also functions in the blood coagulation pathway as well as in the regulation of blood pressure. In addition to plasma prekallikrein deficiency,[14] SNPs of *KLKB1* are significantly associated with venous thromboembolism[1,10] and with the levels of serum metabolomes such as lipids, carbohydrates, and peptides related to atherosclerosis.[35]

The search for calcified-plaque-specific variants not listed in the dbSNP

The above gene variants were carefully selected using previously obtained information about variants that are likely to be involved in amino acid/protein alterations and to be pathogenic. Herein we attempted to find novel variations that are specific to high- or low-calcified plaques that are not yet in the dbSNP database. Seven mutated genes from our group comparison and 12 genes from the individual comparisons were identified and applied to the functional analysis. Unfortunately, no enrichment for gene variants from the group comparison and no genes with a high-enough enrichment score from the individual comparisons were detected, presumably mainly due to the small sample size. Larger cohort studies might be helpful to address this.

The clinical significance of investigations of calcified carotid plaques

In general, the culprit plaques have been intently investigated because the results may be directly useful in the future for symptom alleviation and preventive treatments for symptomatic plaques. The prior investigations included studies of gene regulation therapies that inhibit promoters of the proliferation of vascular smooth muscle cells or angiogenesis using miRNAs.[2] On the other hand,

the calcified plaques in carotid arteries often seen in asymptomatic cases are considered stable and are thought to induce fewer symptoms. Nandalur *et al.*[21] reported that carotid-plaque calcification >45% of the total volume was significantly inversely associated with the occurrence of symptoms, and Kwee[15] found through a systematic review that clinically symptomatic carotid plaques have a lower degree of calcification than asymptomatic plaques. Hence, carotid calcified plaques have not been as thoroughly investigated compared to the coronary artery calcification that is closely related to CAD.[1]

As demonstrated in the previous studies[11-13] and our present study, however, it is relatively easy to quantitatively and morphologically evaluate calcification with calcium scores by MDCTA compared to an analysis for vulnerable soft plaques. Information about cellular and genetic stabilizing mechanisms obtained by analyses of "stable" calcified carotid plaques (as in the present study) may lead to more insights that will help clinicians cope with "unstable" plaques. If plaques are quantitatively and precisely classified using calcium scores, the information about a less-calcified soft-plaque group (like *KLKB1* in the present study) could also be harvested. Although it would be challenging to apply the present results directly to the control of genetic activity in carotid plaques to prevent symptoms, they could contribute to the understanding of the molecular mechanisms underlying the stability versus vulnerability in high- and low-calcified plaques and future therapeutic strategies.

Limitations

The main limitation of the study is the small sample number. In addition, we cannot clearly declare whether the gene variations extracted herein were somatic or germline-derived. We harvested the genome from plaques with the acquired atheromatous change, not from peripheral blood, as in the prior SNP studies for rare variants. We may have thus more opportunities to obtain somatic-prone genetic variations. We also excluded the variants with $0.4 \leq AF \leq 0.6$ that were likely to have a germline origin in order to narrow down the genetic information to somatic information as much as possible, avoiding extra invasive interventions to the patients. More precise information would have been obtained, however, by collecting additional peripheral blood samples or other normal tissues and comparing them with the carotid plaque results. We are designing a further investigation in consideration of these points.

CONCLUSION

Our findings demonstrated the precise profile of base-pair substitutions in carotid plaques. No significant differences were detected between the high- and low-calcified plaques. However, genetic variants in *ADCC6* relating to vascular calcification for high-calcified plaques, and in *KLKB1* encoding kallikrein associated with vascular regulation of atherosclerosis for low-calcified plaques were more frequently discovered compared to the other group. These results may contribute to a better understanding of the genetic signatures of calcium modulation and cellular activity in carotid plaques.

Declaration of patient consent

Institutional Review Board permission obtained for the study.

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Conflicts of interest

There are no conflicts of interest.

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