Original Article

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Forkhead box O3 longevity genotype may attenuate the impact of hypertension on risk of intracerebral haemorrhage

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Objective: Since the *G* allele of forkhead box O3 (*FOXO3*) single nucleotide polymorphism (SNP) *rs2802292* is associated with resilience and longevity, ostensibly by mitigating the adverse effects of chronic cardiometabolic stress on mortality, our aim was to determine the association between the *FOXO3* SNP *rs2802292* genotype and risk of hypertension-mediated intracerebral haemorrhage (ICH).

Methods: From a prospective population-based cohort of Japanese American men from the Kuakini Honolulu Heart Program (KHHP), age-adjusted prevalence of ICH by hypertension was assessed for the whole cohort after stratifying by *FOXO3* genotype. Cox regression models, adjusted for age, cardiovascular risk factors and, *FOXO3* and *APOE* genotypes, were utilized to determine relative risk of hypertension's effect on ICH. All models were created for the whole cohort and stratified by *FOXO3 G*-allele carriage vs. *TT* genotype.

Results: Among 6469 men free of baseline stroke, *FOXO3 G*-allele carriage was seen in 3009 (46.5%) participants. Overall, 183 participants developed ICH over the 34-year follow-up period. Age-adjusted ICH incidence was 0.90 vs. 1.32 per 1000 person-years follow-up in those without and with hypertension, respectively ($P = 0.002$). After stratifying by *FOXO3* genotype, this association was no longer significant in *G* allele carriers. In the whole cohort, hypertension was an independent predictor of ICH (relative risk [RR] = 1.70, 95% confidence interval [CI] 1.25, 2.32; $P = 0.0007$). In stratified analyses, hypertension remained an independent predictor of ICH among the *FOXO3 TT*-genotype group (RR = 2.02, 95% CI 1.33, 3.07; $P = 0.001$), but not in *FOXO3 G*-allele carriers (RR = 1.39, 95% CI 0.88, 2.19; $P = 0.15$).

Conclusions: The longevity-associated *FOXO3 G* allele may attenuate the impact of hypertension on ICH risk.

Keywords: forkhead box O3, hypertension, intracerebral hemorrhage, stroke

Abbreviations: APOE, apolipoprotein E; CAD, coronary artery disease; CBA, Charcot-Bouchard aneurysms; CT, computed tomography; FOXO3, forkhead box O3; ICH, intracerebral haemorrhage; MRI, magnestic resonance imaging; PAI, physical activity index; SAH, subarachnoid haemorrhage; SNP, single-nucleotide polymorphism

INTRODUCTION

Minor alleles of multiple single nucleotide polymorphisms (SNPs) located in the forkhead box O3 (*FOXO3*) gene (particularly the *G* allele of SNP *rs2802292*) have been strongly associated with human longevity in multiple studies [1–3]. In the Kuakini Honolulu Heart Program (KHHP) cohort, the presence of the longevity-associated *FOXO3* allele was associated with increased likelihood of living to almost 100 years [4]. Furthermore, the presence of longevity-associated *FOXO3* alleles confers association with protection against mortality from coronary artery disease (CAD) [5]. Although the exact mechanism by which *FOXO3* genotype is associated with healthy aging and increased lifespan is unclear, it has been postulated that the longevity-associated *FOXO3 G*-allele may serve as a 'resilience' gene by mitigating the adverse effects of chronic cardiometabolic stress on intracellular processes, thereby reducing the risk of life-threatening cardiovascular events [3,6].

Intracerebral haemorrhage (ICH) results in haemorrhagic stroke which has high morbidity and mortality [7]. Although hypertension is the major risk factor for ICH [8],

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the presence of apolipoprotein E (*APOE*) ε2 and/or ε4 alleles, as opposed to the more common ε3/ε3 genotype, has also been associated with increased risk of ICH, predominantly with lobar ICH [9–11]. A recent genome-wide association study (GWAS) has identified several genetic loci that are associated with increased risk of ICH [12]. Although *FOXO3* was not identified in the GWAS as an ICH-associated gene, *FOXO3* may nevertheless have a protective role against ICH by reducing the cumulative burden of hypertension on cerebral vessels. Assessing gene–environment interaction can improve understanding of how a known genotype, in this case *FOXO3* genotype, may modulate the end-organ manifestation of a chronic disease [13]. To date, the impact of the longevity-associated *FOXO3 G*-allele on hypertension-related risk of ICH has not been studied. We hypothesized that the *FOXO3 G*-allele might attenuate the impact of hypertension on ICH risk (Fig. 1). Ours is the first longitudinal study to examine the interaction between hypertension and *FOXO3* genotype on risk of ICH.

METHODS

Study design and participants

The KHHP is a prospective population-based study of cardiovascular disease among Japanese-American men living in Hawaii. From 1965, the KHHP began following 8006 men of Japanese ancestry living on the island of Oahu for the development of CAD and stroke [14–16]. Participants were identified using World War II Selective Service Registration files. They were 45–68 years old at baseline examination between 1965 and 1968. They have been followed since then with periodic examinations, and continuous hospital surveillance for selected morbidity and all mortality through December 1999. After excluding those with prevalent stroke at baseline and those with missing *FOXO3* genotype data, our analytical sample comprised 6469 men. Procedures performed were in accord with institutional guidelines and were approved by the Institutional Review Board of Kuakini Medical Center. Written informed consent was obtained at all examination cycles.

Data collection

Data on cardiovascular risk factors were obtained at the baseline examination (1965–1968).

Hypertension was defined as systolic/diastolic blood pressure ≥140/90 mmHg or taking antihypertensive medications. Body mass index was defined as weight in kilograms divided by height in meters squared. Diabetes was defined by history or use of insulin or oral hypoglycaemic medications. Smoking was defined as pack-years by self-report. Physical activity index (PAI) was quantified as metabolic output during a typical 24-h period by multiplying a weighting factor by the number of hours spent in 5 activity levels (no activity = 1.0, sedentary = 1.1, slight = 1.5, moderate = 2.4 and heavy = 5.0) [17]. Serum cholesterol was measured in nonfasting blood samples. Alcohol intake was measured by self-report as ounces per month.

Genotyping

Genotyping of *FOXO3* and *APOE* was performed on blood samples that had been frozen at −70°C. For men who participated in Examination 4 (1991–1993), DNA for genotyping was obtained from the blood sample buffy coat [18]. For other participants, genotyping was performed using DNA obtained from serum frozen at Examination 3 (1971–1974). After DNA isolation, PCR was used for amplification of a suitable region of each gene using a combination of QIAmp cell-free DNA isolation followed by REPLI-g Single-Cell WGA & WTA amplification (QIAGEN Sciences, Germantown, Maryland, USA). Genotyping was performed using TaqMan on an Applied Biosystems QuantStudio 12K Flex system (ThermoFisher Scientific, Waltham, Massachusetts, USA).

Outcome measures

The cohort has undergone continuous surveillance for all mortality and selected morbidity parameters, including stroke, from 1965 to December 1999. All hospital discharge records on the island of Oahu, death certificates and autopsy records were reviewed. Surveillance for this cohort is

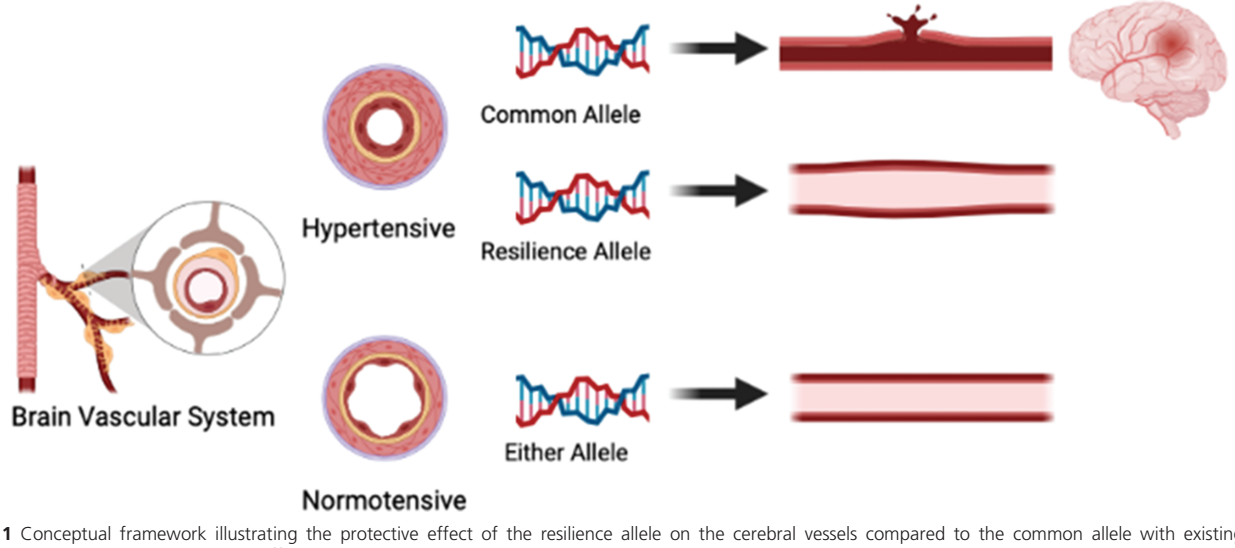

FIGURE 1 Conceptual framework illustrating the protective effect of the resilience allele on the cerebral vessels compared to the common allele with existing chronic hypertension. Without hypertension, the effect on the cerebral vessels is comparable between the two alleles.

considered essentially complete. We excluded 113 participants with prevalent stroke at baseline. In the original cohort, incident stroke was defined as the acute onset of a neurological deficit for 2 weeks or until death, confirmed by either blood in the cerebrospinal fluid or evidence from brain computed tomography (CT) or magnetic resonance imaging (MRI). Possible strokes, defined as neurological deficits persisting for at least 24 h but for less than 2 weeks or an unknown duration, were not included as stroke events because of diagnostic uncertainty. Strokes were classified as thromboembolic, haemorrhagic or unknown type based on clinical information and findings of imaging studies, surgery, or autopsy. Haemorrhagic stroke was diagnosed if a focal neurological deficit was associated with loss of consciousness, headache, and blood in the spinal fluid from a traumatic lumbar puncture or based on neuroimaging, surgical, or autopsy findings. Some of the haemorrhagic strokes were diagnosed clinically without any radiographic or autopsy confirmation since they occurred before the advent of CT scanning. Participants with focal neurological findings from other causes such as blood dyscrasias, neoplastic disease, head injury, surgical accident, meningoencephalitis, fat embolism, epilepsy or cardiac arrest were excluded. All stroke diagnoses were originally confirmed by a study neurologist and the Kuakini Honolulu Heart Program Morbidity and Mortality Committee using standardized research criteria (*International Classification of Diseases, 8th Revision* codes 430–438).

For the present study, to differentiate spontaneous ICH from other haemorrhagic strokes, all original cases of incident 'haemorrhagic stroke' were reviewed retrospectively, including ICH, spontaneous subarachnoid haemorrhage (SAH), haemorrhagic transformation of ischaemic stroke, and traumatic intracranial haemorrhage. A board-certified neurologist and neurointensivist (K.N.) reviewed all cases that were initially classified as haemorrhagic stroke to assess the accuracy of the diagnosis, predominantly using the imaging and autopsy reports. Specifically, determination of ICH was made when the radiographic or autopsy report excluded traumatic intracranial haemorrhage, ruptured cerebral aneurysm, or ischaemic stroke with haemorrhagic transformation. Since the KHHP began before the use of CT scanning, some ICH cases that did not have any associated neuroimaging findings and/or lacked autopsy data were excluded from the analyses. Only cases with confirmatory neuroimaging or autopsy reports that described the location and the pattern of the haemorrhage to support the diagnosis of spontaneous ICH were included in the analyses for this study. Due to the limited number of original neuroimaging studies available to review and our attempt to speculate on the ICH location (lobar vs. deep) based on the written radiology report, ICH location was not included in the data analysis.

Statistical analysis

Mean age-adjusted baseline risk factor levels were compared among participants with the *FOXO3 TT* genotype and carriers of the *FOXO3 G*-allele using General Linear Models (GLM). Kaplan–Meier survival curves were created to compare ICH disease-free survival among those with and without hypertension, stratified by *FOXO3* genotype. Age-adjusted incidence of ICH per 1000 person-years follow-up over a 34-year period was assessed for those with and without hypertension for the whole cohort and after stratifying by *FOXO3* genotype. Cox proportional hazards models, adjusted for age, cardiovascular risk factors, and *FOXO3* and *APOE* genotypes, were used to assess the relative risk of impact of hypertension on ICH incidence. All models were created for the whole cohort and then stratified by *FOXO3* genotype (*G*-allele carriage vs. *TT* genotype). The Cox proportional hazard assumption was tested for each Cox model. All statistical analyses were performed using the Statistical Analysis System (SAS) version 9.4 (Cary, North Carolina, USA). Power analysis was performed and values were generated using StataCorp. 2019 Stata Statistical Software Release 16 (College Station, Texas, USA).

RESULTS

Among a total of 8006 participants, 113 with a baseline history of stroke and 1332 with absent *FOXO3* genotype were excluded. There were 92 participants who later developed haemorrhagic stroke but who did not meet the study criteria for spontaneous ICH and were also excluded. In the final analyses, the data from 6469 men were utilized. *FOXO3 G*-allele carriage was present in 3009 (46.5%) participants. Overall, 183 participants developed ICH over the 34-year follow-up period.

The age-adjusted baseline characteristics of the cohort comparing those with *FOXO3 TT* genotype and those with possession of one or two *G*-alleles are shown in Table 1.

Kaplan–Meier survival curves demonstrated a significant difference in the 34-year ICH-free survival in those with and without hypertension in the whole cohort (log rank $P = 0.0006$) and in the *FOXO3 TT* genotype group (log rank $P = 0.0015$), but not in the *FOXO3 G*-allele carriers (log rank $P = 0.103$) (Fig. 2).

Age-adjusted prevalence of incident ICH per 1000 person-years follow-up are shown in Table 2. Incident ICH prevalence was significantly higher among participants with prevalent hypertension than those without prevalent hypertension in the whole cohort (1.32 vs. 0.90 per 1000 person-years follow-up, respectively; $P = 0.0024$) and among those with *FOXO3 TT* genotype (1.39 vs. 0.80 per 1000 person-years follow-up, respectively; $P = 0.0052$), but not among those who were *FOXO3 G*-allele carriers (1.24 vs. 0.98 per 1000 person years follow-up respectively; $P = 0.15$).

Using multivariate Cox regression in the whole cohort, hypertension was an independent predictor of ICH (relative risk [RR] = 1.70, 95% confidence interval [CI] 1.25, 2.32; $P = 0.0007$). The significance of the interaction term between hypertension and *FOXO3* genotype was tested by comparing the log likelihood ratio of the full regression model, including hypertension and *FOXO3* genotype cross-product, vs. the reduced model without the cross-product term. The interaction effect of hypertension and *FOXO3* genotype was −0.34 (log HR; $P = 0.26$), and indicated a moderate interaction. Since the effects of hypertension on ICH incidence may be different for different *FOXO3* genotypes, we performed stratified

TABLE 1. Mean baseline risk factor levels for contrasting *FOXO3* genotypes

Baseline risk factor *Age-adjusted	*FOXO3 TT* *n* = 3460	*FOXO3 G-allele* *n* = 3009	*P* value
Age (years)	53.8 ± 5.38	54.1 ± 5.43	0.047
Prevalent hypertension (%)*	38.9%	38.8%	0.98
BMI (kg/m^2)*	23.9 ± 3.02	23.8 ± 3.00	0.26
Prevalent type 2 diabetes (%)*	8.55%	8.78%	0.74
Smoking (pack-years)*	22.6 ± 23.5	22.7 ± 23.9	0.88
Physical activity index*	32.9 ± 4.6	32.8 ± 4.4	0.29
Total cholesterol (mg/dl)*	218.2 ± 37.1	218.5 ± 36.2	0.75
Alcohol intake (oz/month)*	13.8 ± 24.7	12.8 ± 21.9	0.076
APOE ε2 or ε4*	26.48%	26.25%	0.84

Hypertension = SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg or use of antihypertensive medications.
Diabetes = medical history, with and without use of medications.

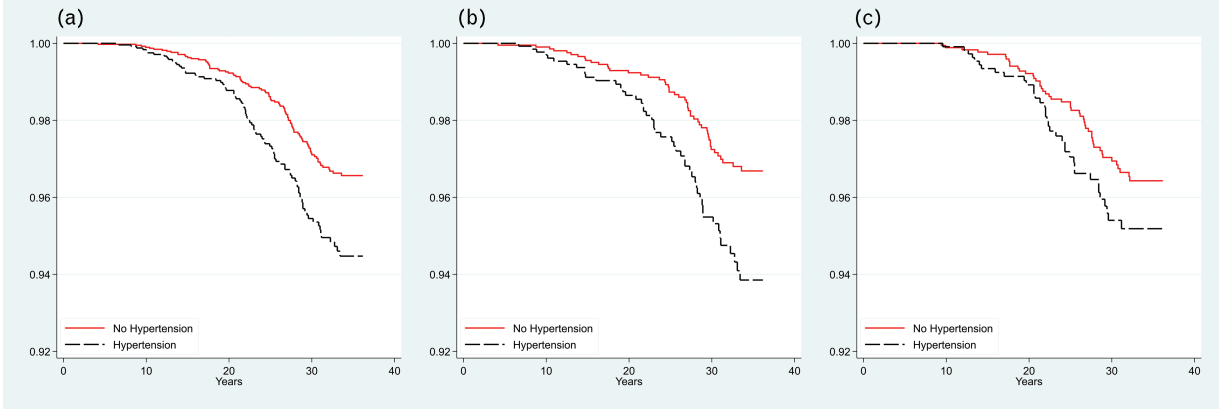

FIGURE 2 Kaplan–Meier curves demonstrating intracerebral haemorrhage (ICH)-free survival with and without prevalent hypertension, stratified by *FOXO3* genotype for: (a) entire cohort ($n = 6469$; log-rank $P = 0.001$); (b) *FOXO3 TT* genotype only ($n = 3460$; log-rank $P = 0.002$); (c) *FOXO3* (any *G* allele only) ($n = 3009$; log-rank $P = 0.103$).

TABLE 2. Age-adjusted rates of incident intracerebral haemorrhage (ICH) (per 1000 person years) without and with prevalent hypertension, stratified by *FOXO3* genotype

Incident ICH rates (*n*)	Prevalent hypertension No, *n* = 3946	 Yes, *n* = 2523	*P* value
Entire cohort ($n = 6469$)	0.90 (97/3946)	1.32 (86/2523)	0.002
Stratified analysis: *FOXO3 TT* genotype ($n = 3460$)	0.80 (49/2115)	1.39 (49/1345)	0.005
Stratified analysis: *FOXO3* any *G* allele ($n = 3009$)	0.98 (48/1831)	1.24 (37/1178)	0.15

analyses for different *FOXO3* genotypes. Table 3 shows that hypertension remains as an independent predictor of ICH among the *FOXO3 TT* genotype group (RR = 2.02, 95% CI 1.33, 3.07; $P = 0.0010$), but not among *FOXO3 G*-allele carriers.

DISCUSSION

Among participants homozygous or heterozygous for the longevity-associated *G*-allele of *FOXO3* SNP *rs2802292*, the impact of hypertension on the 34-year risk of ICH

TABLE 3. Cox regression analysis results showing relative risks of prevalent hypertension for incident intracerebral haemorrhage (ICH), stratified by *FOXO3* genotype

	Entire cohort ($n = 6469$)		*FOXO3* (*TT*) ($n = 3460$)		*FOXO3* (*TG/GG*) ($n = 3009$)	
	RR (95% CI)	*P*	RR (95% CI)	*P*	RR (95% CI)	*P*
Model 1	1.65 (1.24–2.21)	<0.001	1.88 (1.27–2.80)	0.002	1.43 (0.93–2.19)	0.10
Model 2	1.70 (1.25–2.31)	<0.001	2.01 (1.32–3.06)	0.001	1.40 (0.89–2.21)	0.14
Model 3	1.70 (1.25–2.32)	<0.001	2.02 (1.33–3.07)	0.001	1.39 (0.88–2.19)	0.15

Model 1 – Prevalent hypertension.
Model 2 – Prevalent hypertension, adjusted for age and CVD risk factors*.
Model 3 – Prevalent hypertension, adjusted for age, CVD risk factors, *FOXO3* and *APOE* genotype.
Genotypes: The interaction effect (−0.34) of *FOXO3* (*TG/GG*) and hypertension (+) on ICH was estimated by adding the interaction term, *FOXO3* × hypertension, to model 3 and $P = 0.26$.

was modestly attenuated compared with those lacking the longevity-associated *FOXO3* genotype. We speculate that longevity-associated *FOXO3* variants exert an effect on cerebrovascular resilience so as to protect against the adverse effects of chronic hypertension. Comparable *FOXO3* genotype-related resilience to chronic cardiovascular stress was shown previously in KHHP participants having a cardiometabolic disease (CMD) [6].

In primary ICH, hypertension is thought to be the underlying cause in 65% of cases, followed by cerebral amyloid angiopathy (CAA) [19]. Although hypertension is a well known risk factor for ICH, the pathogenesis of ICH from hypertension is unclear. Chronic hypertension may lead to cerebral small vessel disease (SVD), which then induces degenerative changes in the penetrating arterioles. The changes include fibrinoid necrosis and deposition of plasma proteins such as fibrin in the arteriolar wall, with accompanying degeneration of smooth muscle cells and formation of Charcot-Bouchard aneurysms (CBA) or microaneurysms [20–23]. Although the involvement of CBA in ICH has been challenged recently [24], it remains a plausible pathophysiological explanation for ICH. Three types of CBA were described by Fisher in patients with hypertension, multiple small infarcts, and/or massive cerebral haemorrhage and severe atherosclerosis: saccular, asymmetric fusiform, and lipohyalinotic CBA [25]. Fisher has used the term lipohyalinosis to describe the segmental fibrinoid necrosis of the arterioles with fatty changes from foamy macrophages in the cerebral arterioles [23].

Fibrinoid necrosis is produced by the insudation of plasma fibrin or fibrinogen that are then converted to fibrin in the arteriolar wall. Since the risk of hypertensive haemorrhages is proportional to the blood pressure level [26], one would expect that there may be a direct relationship between the blood pressure level and the severity of fibrinoid necrosis. However, some studies have found fibrinoid necrosis in those who only had mild or benign hypertension, suggesting that other factors may influence the susceptibility of brain arterioles to development of fibrinoid necrosis in response to chronically elevated blood pressure [22].

It has been proposed that the *FOXO3* transcription factor may protect blood vessels by effects on pathways that result in inhibition of vascular smooth muscle cell proliferation and neointimal hyperplasia [27], and thereby provide protection from vascular ageing processes [3]. Activation of *FOXO3* transcription in human embryonic stem cells resulted in reinforcement of human vascular cell homeostasis, delayed ageing, and increased resistance to oxidative injury compared with wild-type cells [28]. Loss-of-function studies have shown that *FOXO3* helps to maintain homeostasis of a diverse array of vascular cell types [29,30]. We therefore hypothesize that *FOXO3* may protect against fibrinoid necrosis in cerebral arterioles of patients with chronic hypertension. Future studies are needed to further assess how *FOXO3* and its encoded protein may impact fibrinoid necrosis formation in cerebral arterioles in response to chronic hypertension.

We acknowledge that our study has some limitations. Given the effect of the interaction and the covariant variables in the Cox model, the power of the test for interaction term was estimated to be 0.20, indicating that our analysis was under-powered. A power analysis suggests that 1165 ICH amongst the 6469 participants (18% cases) would have been needed to reach sufficient power. We believe the small number of ICH events (183), a relatively uncommon event in this cohort, is one of the reasons for the moderate interaction observed. Our study population of Japanese-American men in Hawaii limits generalizability of the study findings to other ethnic groups and to women. Since haemorrhagic stroke events without neuroimaging and/or autopsy results were excluded from the study, this could have led to over-exclusion of true ICH cases. Since possible strokes that had neurological deficits lasting for <2 weeks were excluded based on the original study criteria, a mild ICH may have been inadvertently excluded from the study. Given the lack of complete and accurate data on ICH location, the impact of genotypes such as *APOE* and *FOXO3* on lobar haemorrhage could not be assessed. The study did not include women, the reason being that in 1965–1968 when recruitment took place, heart disease was uncommon in middle-aged women. However, this study also has many strengths, including a large overall sample size, its prospective study design, and the very long follow-up period. Although the cohort only included Japanese-American men, the population is unique in that it is genetically more homogenous than other racial populations and has not been studied extensively. We had considerable data on other cardiovascular risk factors, allowing us to adjust for these factors to minimize confounding. Our surveillance system for incident stroke was thorough, given that this was an island population. Although the interaction effect was modest, the study is important because it is the first to examine the possible effect of *FOXO3* genotype on the relationship between chronic hypertension and ICH.

In conclusion, the present study found that *FOXO3* longevity genotype may attenuate the impact of hypertension on the risk of intracerebral haemorrhage in Japanese-American men in Hawaii. Future studies should attempt to replicate these findings in larger populations elsewhere, including in other ethnic groups, and in women.

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Conflicts of interest

There are no conflicts of interest.

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