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

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\textbf{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).

\textbf{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.

\textbf{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; \(P = 0.15)\).

\textbf{Conclusions:} The longevity-associated FOXO3 G allele may attenuate the impact of hypertension on ICH risk.

\textbf{Keywords:} forkhead box O3, hypertension, intracerebral hemorrhage, stroke

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

\textbf{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 \([8]\,\ldots\)
the presence of apolipoprotein E (APOE) \(\varepsilon2\) and/or \(\varepsilon4\) alleles, as opposed to the more common \(\varepsilon3/\varepsilon3\) 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 \(\geq140/90\) 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 \(-70^\circ\)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
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).

In 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 1352 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...
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
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 hemorrhage 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 [25].

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 hemorrhage 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 homoeostasis, 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 homoeostasis 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.

ACKNOWLEDGEMENTS

The authors thank all study participants and their families for their cooperation and the Hawaii State Department of Health for its help. The authors wish to acknowledge Dr Alvin T. Onaka, Brian Horiiuchi, and Caryn Tottori of the Hawaii State Department of Health for providing death certificate data on cause of death for the KHHP participants, Ms. Ayako Elliott and Ms. Eva Ardo for assistance with genotyping, and Ms. Hiromi Nakada and Ms. Ka-on Fong for monitoring the vital status of KHHP participants.

Sources of Funding: This work was supported by the Kuakini Medical Center, the US National Institutes of Health (contract N01-AG-4–2149, Grants 5 U01 AG019349-05, 5R01AG027060 [Kuakini Hawaii Lifespan Study], 5R01AG038707 [Kuakini Hawaii Healthspan Study], 1P20GM125526-01A1 [Kuakini Center of Biomedical Research Excellence for Clinical and Translational Research on Aging]), and contract N01-HC-05102 from the National Heart Lung and Blood Institute.
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Conflicts of interest
There are no conflicts of interest.

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