Discussion

This is the first report that has compared sFas levels among potential cancer cases from all sites and controls in a large-scale prospective study. We found that a higher sFas level was associated with an increased risk for cancer mortality, even among individuals whose cancer death occurred in the latter period (6.4 ± 1.4 years on an average after blood donation).

There have been several cross-sectional studies that compared sFas levels between cancer cases and healthy volunteers. Compared with those of controls, sFas levels are higher in the serum of cases with melanoma,[5] non-Hodgkin's lymphoma,[7] ovarian cancer,[3,12] cervical and endometrial cancer,[12] breast cancer,[2] hepatocellular carcinoma,[13] renal cell carcinoma[4] and bladder cancer.[1] Among cancer cases, some studies revealed sFas levels showed correlations with disease survival.[1-3,5,7,12] It was also confirmed in several studies that surgical removal of tumors reduced sFas levels.[4,13,14] As Holdenrieder and Stieber described in their review,[15] cancer itself has some self-defense mechanisms for avoiding apoptosis, one of which must be sFas.

Using stored serum from the participants of a large-scale cohort study before cancer diagnosis, we found a clear association between sFas levels and cancer mortality. This finding suggested that sFas could not only be a prognostic factor in patients with cancer but also a valid biomarker to identify people at high risk for cancer prior to diagnosis. Moreover, it is likely that cancer progression might be expedited and its prognosis worsened if cancer occurs in those with high serum sFas levels, because the risk elevation was almost the same in each observation period. Although the biological mechanisms that might explain the findings of this study have not been clarified yet, there is a possibility that cancer cells release sFas to avoid apoptosis even at the very early stage. However, from a nested case–control study in which cases and controls were selected from 3 cohort studies, Akhmedkhanov *et al.* failed to find any association between serum sFas drawn an average of 5.1 years before diagnosis and the incidence of ovarian cancer.[6] This contrast to the result from our study might be due to differences in the treated outcome [incidence and mortality, and ovarian cancer and cancer of all sites].

Our study has several strengths. We examined the associations between sFas levels and the risk of total cancer mortality in a prospective, nested case–control study. Sera were collected at an average of 4.7 years before cancer mortality. The prospective design could avoid the problems of questionable temporal relationships between sFas levels and cancer risks that hamper traditional case–control studies. In addition, a large number of cases were accumulated during the long follow-up period. Data on confounding factors were available, and their potential effects could be controlled by using multivariable analyses.

We also have to consider some limitations of our study when interpreting the results. First, since not all the cohort participants provided blood samples, the possibility of a selection bias could not be excluded. However, at the time of blood donation, no one could anticipate the subsequent cancer occurrence or mortality. Since cases and controls underwent the same process of selection before blood donation, that donation depended solely on the subject's intention. Thus, any bias due to blood donation or selection of cases and controls would not seriously affect our results. Second, serum samples were stored for ~10 years in deep freezers at −80°C. The stability of sFas in these cohort samples could not be determined because their values were not measured at baseline. However, Ito *et al.* compared newly collected sera and frozen specimens stored for 9 years, gathered from a variety of different individuals, and found no statistically significant difference in the distributions of sFas values,[11] indicating that the serum sFas level remained stable after long-term storage at −80°C. Furthermore,

TABLE II – CASE AND CONTROL DISTRIBUTION OF SOME DEMOGRAPHIC FACTORS

	Controls	Cases
Total number	2,353	798
Gender		
Men (%)	57.8	57.6
p-value	0.87	
Age at baseline		
40–49 (%)	5.1	5.4
50–59 (%)	20.9	20.6
60–69 (%)	46.2	45.6
70–79 (%)	27.8	28.4
Mean	64.2	64.3
SD	8.0	8.1
p-value*	0.90	
Smoking habits		
Current smoker (%)	27.0	37.2
p-value**	<0.001	
Drinking status		
Current drinker (%)	50.8	47.6
p-value**	0.08	
Body mass index		
<18.5 (%)	5.9	8.2
18.5<=, <25 (%)	75.7	72.1
25<= (%)	18.4	19.7
Mean	22.6	22.6
SD	3.1	3.0
p-value**	0.73	

p, performed by Mantel Haenszel test adjusted for area and age category.
**p*, performed by Mantel Haenszel test adjusted for area and gender.
***p*, performed by Mantel Haenszel test adjusted for area, gender and age category.

TABLE III – ODDS RATIOS OF CANCER MORTALITY ACCORDING TO sFAS LEVEL

	Control	Case	OR1	95% CI	Trend *p*	OR2	95% CI	Trend *p*
Total								
Q1	579	151	1.00			1.00		
Q2	627	188	1.20	0.94–1.54		1.17	0.91–1.51	
Q3	534	179	1.39	1.08–1.80		1.32	1.02–1.71	
Q4	613	280	1.96	1.52–2.52	0.005	1.81	1.40–2.34	0.007
Before 1994								
Q1	293	78	1.00			1.00		
Q2	319	95	1.19	0.84–1.67		1.19	0.84–1.68	
Q3	284	88	1.27	0.89–1.83		1.21	0.83–1.74	
Q4	342	159	1.97	1.39–2.80	0.031	1.81	1.26–2.58	0.042
After 1994								
Q1	286	73	1.00			1.00		
Q2	308	93	1.22	0.85–1.74		1.16	0.81–1.67	
Q3	250	91	1.53	1.06–2.21		1.44	0.99–2.09	
Q4	271	122	1.93	1.34–2.77	0.056	1.81	1.25–2.62	0.053

OR1, adjusted for age.
OR2, adjusted for age, BMI, smoking and drinking status.
sFas level: Q1 < 1.9; Q2, 1.9–2.2; Q3, 2.3–2.6; Q4, 2.7<= ng/ml.

the average 8.2% difference in sFas values between sera collected from 100 individuals in 1991 and in 1999 reported by Ito *et al.*[11] was similar to the coefficients of variation for determinations: 2.1–5.5% for intraassay and 8.2–12.3% for interassay. Third, although we have made some adjustment for possible confounders, there were some diseases, such as autoimmune disease, that might influence the level of sFas.[16] Unfortunately, it was impossible to adjust for these factors because of lack of information, however, if such diseases occurred at random, the estimated OR might approach null. In conclusion, our results suggest the possibility that serum sFas could indeed be a predictive marker of latent cancer before its diagnosis, since it increased in serum from apparently healthy people drawn an average 4.7 years before mortality.

Acknowledgements

The authors express their sincere appreciation to Dr. Kunio Aoki, Professor Emeritus, Nagoya University School of Medicine and a former chairperson of the JACC Study, to Dr. Haruo Sugano, the former Director of the Cancer Institute, Tokyo, who greatly contributed to the initiation of the JACC Study, and to Dr. Yoshiyuki Ohno, Professor Emeritus, Nagoya University School of Medicine, who was also a former chairperson of the study. The authors also thank Dr. Tomoyuki Kitagawa of the Cancer Institute of the Japanese Foundation for Cancer Research and a former chairperson of Grant-in-Aid for Scientific Research on Priority Area "Cancer," for his valuable support of this study.

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Exon 19 of *EGFR* mutation in relation to the CA-repeat polymorphism in intron 1

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(Received December 22, 2007/Revised January 27, 2008/Accepted January 31, 2008/Online publication April 14, 2008)

Epidermal growth factor receptor *(EGFR)* mutations in lung cancer enhance tyrosine kinase activity and increase sensitivity to the EGFR tyrosine kinase inhibitor, gefitinib. Mutation analysis of the *EGFR* gene is therefore indispensable for predicting gefitinib response. We investigated a CA-repeat polymorphism in the *EGFR* gene related to *EGFR* mutations. Because an increasing number of CA-repeats at intron 1 of the *EGFR* gene has been reported to reduce transcription activity, we examined the relationship between *EGFR* mutations and this CA-repeat polymorphism. *EGFR* mutations at exon 19 were closely associated with shorter CA-repeat length in the shorter allele, but this was not the case for *EGFR* mutations at exons 18 or 21. Increased intrinsic *EGFR* mRNA expression in non-cancerous lung tissues from lung adenocarcinoma patients was also significantly associated with shorter CA-repeat length. A higher frequency of *EGFR* mutations at exon 19 was associated with shorter CA-repeat length only in patients with high levels of *EGFR* mRNA expression. To determine the phenotypes of cells possessing shorter CA-repeats, an *in vitro* study using human bronchial epithelial cells with different CA-repeat lengths was performed; more rapid cell growth and activated EGF/EGFR signaling were found more often in the cells having both shorter CA-repeats and increased *EGFR* mRNA expression. These results suggest that CA-repeat length in the *EGFR* gene may be a genetic factor related to cancer in the case of *EGFR* mutations at exon 19. The mechanism likely involves enhanced intrinsic expression of *EGFR* mRNA and activated EGF/EGFR signaling that accompany shorter CA-repeats. (*Cancer Sci* 2008; 99: 1180–1187)

Lung cancer is the most common form of cancer death among males and the third most common among females in Japan.[1] Overall survival from lung cancer remains unsatisfactory, with a five-year survival rate of 14%, indicating that this is one of the most difficult cancers to treat.[2] However, the therapeutic strategy has changed since molecular targeting therapies, such as the use of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, became available. Specifically, mutations in the *EGFR* gene found in lung cancer tissues have been reported to be a predictive marker for clinical response to EGFR tyrosine kinase inhibitor therapy, which may lead to the development of customized therapy.[3–6]

Mutations in the *EGFR* gene were found in 30–40% of all lung cancer patients in Japan, and were found in over 50% of female non-smokers with adenocarcinomas.[7–10] Although the overall rate of response to EGFR tyrosine kinase inhibitors for previously treated patients was 27.5% among Japanese,[11] the frequency of gefitinib responsive patients among those with *EGFR* mutations was approximately 80%.[5,9] Therefore, it is clear that DNA sequence analysis of *EGFR* in lung cancer is essential for predicting response to EGFR tyrosine kinase inhibitor therapy. In addition, *EGFR* mutations have been reported to be involved in lung carcinogenesis; an investigation using transgenic mice carrying the *EGFR* mutants $EGFR^{L858R}$ and $EGFR^{\Delta L747-S752}$ demonstrated that mutant *EGFR* was required for the development and maintenance of lung adenocarcinomas.[12] Identification of clinical and genetic factors underlying *EGFR* mutations will be particularly meaningful not only for understanding lung carcinogenesis in non-smokers but also for making clinical decisions about the use of EGFR tyrosine kinase inhibitors in non-operative patients.

The *EGFR* gene is located on chromosome 7p12.1–12.3 in humans, and its expression is regulated by one promoter region and two enhancer regions.[13] The promoter region contains a GC-rich sequence without the characteristic TATA and CAAT boxes, and multiple transcription start sites exist. At least four Sp1 binding sites and one TC factor binding site are known, and basal transcription is regulated by Sp1.[14,15] Two enhancer elements, one located upstream of the promoter (−1429/−1109) and one located downstream at intron 1 (+1788/+2318), function cooperatively.[16] A polymorphic simple sequence repeat with 14–21 CA-repeats was first demonstrated close to the downstream enhancer by Chi *et al.*[17] An increased number of CA-repeats was reported to be associated with decreased *EGFR* transcription activity: a 2-fold increase in transcription activity with 16 CA-repeats compared to that with more than 18 CA-repeats.[18] An *in vitro* run-off assay using a 4050 bp polymerase chain reaction (PCR) product of the *EGFR* gene showed that the level of *EGFR* transcription was reduced by 80% with 21 CA-repeats compared to that with 16 CA-repeats. Using competitive reverse transcription-polymerase chain reaction (RT-PCR), it was demonstrated that pre mRNA expression levels in various cancer cell lines were correlated with the number of CA-repeats; higher levels of *EGFR* mRNA were found in cancer cells with lower numbers of CA-repeats, which is consistent with the *in vitro* experiments.

The relationship between lung cancer and CA-repeat polymorphism has been studied in terms of lung cancer risk or clinical phenotype, specifically gefitinib responsiveness. A case-control study revealed an inverse relationship between lung cancer risk and the number of CA-repeats in a Caucasian population,[19] although no association with lung cancer risk was reported in a Korean population.[20] A clinical study of 86 patients with advanced non-small-cell lung cancer treated with gefitinib in Korea showed that a low number of CA-repeats was associated with gefinitib responsiveness, although no significant association with *EGFR* mutation status nor *EGFR* expression levels was found in cancer tissues.[21] On the other hand, it has been reported that shorter CA-repeat length is associated with an increased expression of *EGFR*.[18,22–24] Furthermore, shorter CA-repeat alleles in lung cancer were more likely to be amplified, resulting in more prevalent allelic imbalance at the *EGFR* locus; *EGFR* mutations were found to favor shorter CA-repeat alleles.[25] However, most of these studies were based on cancer cell lines and resected cancer tissues.

We previously reported that *EGFR* mutations at exons 18, 19 and 21 evidenced different clinical profiles, suggesting that

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doi: 10.1111/j.1349-7006.2008.00804.x

EGFR mutations should be analyzed according to the exons at which they occur.[10] In addition, we think that the relationship between CA-repeat polymorphism and intrinsic *EGFR* expression should also be examined in non-cancerous tissues and cultured normal lung epithelial cells, because *EGFR* expression in cancer tissues may be modified by various genetic alterations such as mutations and allelic imbalance. Indeed, in lung cancer tissues with the same CA-repeat status, *EGFR* mRNA levels differed according to whether the *EGFR* allele was wild-type or mutated, and also depended on the *EGFR* copy number.[26]

In the present study, seeking to identify the genetic factors underlying *EGFR* mutations, we investigated the relationship between the CA-repeat polymorphism and *EGFR* mutations according to exon in 154 patients with lung cancer, 70% of which were of pathological stages I and II. We also studied the association between CA-repeat polymorphism and *EGFR* mRNA levels in non-cancerous tissues from 74 lung adenocarcinoma patients being followed in an *in vitro* study of CA-repeat length, and cell growth using normal human bronchial epithelial (HBE) cells with different CA-repeat lengths obtained from 11 lung cancer patients.

Materials and Methods

Tissue and pleural effusion. We studied a total of 154 Japanese patients with lung cancer. We obtained tissue specimens as follows: 123 surgical specimens, 9 transbronchial fiberscopic specimens, 19 pleural effusions from non-resectable lung cancers, two specimens from metastatic lesions of the brain and skin, and one sample of cells from urine. Study patients were admitted to the Saga Medical School Hospital, Saga, Japan, between 2000 and 2007: 142 patients had not received anticancer chemotherapy or thoracic irradiation, and 12 patients were recurrent cases. Clinical stage was determined according to the criteria of the International Union Against Cancer. Histological subtype and tumor content were confirmed by Hematoxylin and Eosin staining with tumor samples; pleural effusion was assessed as class V by a pathologist. *EGFR* mutation status and CA-repeat length were investigated using DNA direct sequencing. All procedures were performed with the informed consent of the patients, and the study was approved by the Saga University Institutional Review Board Committee.

DNA extraction and sequencing analysis. DNA was isolated from freshly frozen lung cancer tissues using a QIAamp DNA mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Mutations at exons 18, 19, and 21 were determined using PCR-based direct sequencing in cancer tissues; numbers of CA-repeats were determined using adjacent, normal (non-cancerous) tissues. The primers used for *EGFR* mutations were previously described.[3,10] The primer sets used for determination of CA-repeat were 5′-CGGCTGTCCGGCCACTGG-3′ (sense) and 5′-CAGCTCAAGGTTGGAATTGTGC-3′ (antisense) (amplicon size: 378 bp). PCR amplification was performed in a 20-μL volume using Discoverase DHPLC DNA polymerase (Invitrogen Inc., CA, USA) at 95°C for 10 min followed by 40 cycles (each cycle at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min), with a final extension at 72°C for 10 min. The amplified products were isolated using Microcon YM-50 (Millipore Inc., MA, USA) and sequenced directly using the Applied Biosystems PRISM dye terminator cycle sequencing method with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

***EGFR* mRNA expression.** Total RNAs were isolated from both lung cancer tissues and non-cancerous tissues using ISOGEN reagent (Nippon gene, Japan). Levels of *EGFR* mRNA were determined by real-time RT-PCR. One μg total RNA was applied to RT with MuLV reverse transcriptase (Roche Molecular Systems, NJ, USA) at 37°C for 60 min. The cDNAs obtained were processed by quantitative SYBR Green real-time PCR. Each 20 μL SYBR Green reaction consisted of 2 μL cDNA, 2 μL 10 × LightCycler-DNA Master SYBR Green I (Roche Diagnostics Corporation, IN), and 1 μM each of forward and reverse primers. *EGFR* specific primers were 5′-GTCTCTTGCCGGAATGTCAG-3′ (sense) and 5′-CTCACCCTCCAGAAGGTTGC-3′ (antisense) (amplicon size: 67 bp), as previously reported.[27] Quantitative PCR was performed on a Light-Cycler V3 System (Roche Diagnostics Corporation, IN, USA) with 60 cycles, using three-stage program parameters for each cycle as recommended by the manufacturer: 2 s at 95°C, 10 s at 62°C, and 15 s at 72°C. A melting curve analysis was run to assess specificity of the amplified PCR products. Quantification focused on the initial exponential phase of amplification above baseline according to the Light-Cycler software. *EGFR* mRNA levels were standardized by *β-actin* mRNA and log-transformed as log(*EGFR* mRNA/*β-actin* mRNA) for both cancer and non-cancerous tissues.

Cell culture and assessment of cell growth. Primary HBE cells were isolated from bronchial mucosal biopsies of eight lung cancer patients with CA 16/15 (repeat number of the longer allele/shorter allele), six patients with CA 20/19, and one patient with CA 8/7. Following isolation, cells were cultured in Keratinocyte Serum-Free Medium® (Gibco BRL, Life Technologies, Inc., Rockville, MD, USA) containing EGF and bovine pituitary extract at 37°C in 5% CO_2 as described previously.[28] Cells were subjected to experiments after three passages. *EGFR* mRNA detection was performed in the same way as described above for tissue samples.

Western blot analysis. Normal HBE cells were first incubated for 24 h in medium without EGF and bovine pituitary extract, then treated with 100 ng/mL EGF (Sigma, Saint Lous, MS, USA). Whole cell lysates were prepared from cells using lysis buffer containing 50 mM Tris-HCL pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 1% TritonX-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 40 mM sodium fluoride, 1 mM sodium orthovanadate, 1 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM phenolmethylsulfonyl fluoride, as reported previously.[12] Protein (50 μg) was separated using a 10% NuPAGE electrophoresis system (NOVEX, San Diego, CA, USA), transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH, USA), blocked with 5% milk at 4°C overnight, and finally reacted with anti-EGFR, Erk1/2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, antiphospho EGFR, or antiphospho Erk1/2 antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA). An ECL kit (Amersham Corp., Arlington Heights, IL, USA) was used for detection.

Statistical analysis. Baseline characteristics of patients with or without *EGFR* mutations were compared using the χ^2 test for categorical data and the Student's *t*-test for continuous data. A logistic regression model was used to evaluate the contribution of CA-repeat to *EGFR* mutations among lung adenocarcinoma patients, considering gender, age, and smoking status as potential confounders. Differences in means of log(*EGFR* mRNA/*β-actin* mRNA) between two groups defined by CA-repeat number (less than 17 vs 17 or more) in the shorter allele were compared using the Student's *t*-test. The effect of CA-repeat length on *EGFR* mRNA expression level was evaluated by categorical regression analysis with optimal scaling using alternation least squares with adjustment for age, gender, and smoking status (SPSS Categories version 11.0, SPSS Inc., Chicago, IL, USA). For this analysis, we categorized the dependent variable into seven ranks with an approximately normal distribution: log(*EGFR* mRNA/*β-actin* mRNA) ≤–2.23, –1.61–0.12, 0.06–1.37, 1.41–2.58, 2.68–3.47, 4.12–5.40, and ≥5.75. Association between CA-repeat length and *EGFR* mutations at exon 19 was assessed using the $fO^{(2)}$ test separately by level of log(*EGFR* mRNA/*β-actin* mRNA), which was classified into two groups according to the median (<2.10, ≥2.10). Cell growth was compared between the two groups of CA-repeat length in normal HBE cells using the Kruskal–Wallis test; doubling times and *EGFR* mRNA levels were compared using the Mann–Whitney test.

Table 1. Characteristics of patients by *EGFR* mutation status

	EGFR mutations		*P*
	Present ($n = 51$)	Absent ($n = 103$)	
Age (mean ± SD, years)	66.5 ± 10.9	67.8 ± 9.6	ns[2]
Gender			
Male	13 (14%)	78 (86%)	$P < 0.001$
Female	38 (60%)	25 (40%)	
Smoking status			
Smoker	12 (13%)	78 (87%)	$P < 0.001$
Nonsmoker	39 (61%)	25 (39%)	
Histology			
Adenocarcinoma	49 (41%)	70 (59%)	$P = 0.02$
Squamous cell carcinoma	1 (4%)	24 (96%)	
Others	1 (10%)	9 (90%)	
Pathological stage[1]			
I	35 (38%)	58 (62%)	ns
II	3 (21%)	11 (79%)	
III	8 (33%)	16 (67%)	
IV	5 (23%)	17 (77%)	

[1]Examination for distant metastasis was not done in one case.
[2]Not significant.
EGFR, epidermal growth factor receptor.

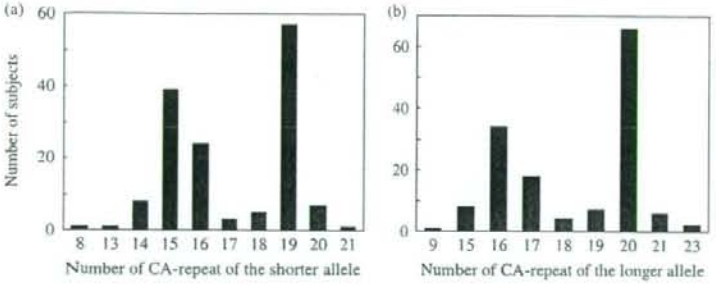

Fig. 1. Distribution of CA-repeat of the shorter allele (a), and the longer allele (b) in lung cancer patients.

All *P*-values presented are two-tailed; those below 0.05 were considered statistically significant. Analyzes were done using SPSS version 12 (SPSS Inc.).

Results

***EGFR* mutations and numbers of CA-repeats.** Clinicopathological characteristics of the 154 patients are shown in Table 1. *EGFR* mutations occurring at exons 18, 19, or 21 were detected in 51 (33%) of the patients. Clear associations of *EGFR* mutations were observed with gender ($P < 0.001$), smoking status (smoker vs non-smoker; $P < 0.001$), and histological type (squamous cell carcinoma, adenocarcinoma, or other; $P = 0.02$). *EGFR* mutations were found in 49 (41%) of 119 adenocarcinoma patients but in only one (4%) of 25 squamous cell carcinoma patients. Distributions of CA-repeat numbers at intron 1 in the shorter and longer alleles of the *EGFR* gene among 146 patients (eight patients were non-informative) revealed two peaks (Fig. 1): CA 19 (39%) and CA 15 (27%) in the shorter alleles and CA 20 (45%) and CA 16 (23%) in the longer alleles.

***EGFR* mutations and CA-repeats.** Because most (96%) of the *EGFR* mutations were found in adenocarcinoma patients, we investigated the relationship between *EGFR* mutations and CA-repeat length only in those patients; 113 patients were examined (we were unable to determine both CA-repeat number and *EGFR* mutation status in six patients). *EGFR* mutations at exons 18, 19, and 21 were detected in 3 (3%), 22 (20%), and 23 (20%), respectively, of the adenocarcinoma patients. The mutations at exon 21 were L858R in 21 patients and L861Q in one patient. All mutations at exon 19 were in-frame deletions with sizes ranging from 9 to 24 base pair.

When study patients were divided into two groups according to the median CA-repeat length (<17 or ≥17) in the shorter allele, *EGFR* mutations at exon 19 were more frequently found in the patients with shorter CA-repeat length ($P = 0.02$, Table 2). On the other hand, *EGFR* mutation frequencies at exons 18 and 21 did not significantly differ with CA-repeat length (Table 2). Logistic regression analysis confirmed the significant contribution of CA-repeat to *EGFR* mutations at exon 19 among the lung adenocarcinoma patients ($P = 0.02$), independently of gender, age, and smoking status (data not shown). In contrast, the same analysis for overall (at exons 18, 19, and 21 combined) *EGFR* mutations in all 154 lung cancer patients demonstrated statistically significant associations with gender and smoking status, but not with CA-repeat length, and the analysis for overall *EGFR* mutation frequency only in lung adenocarcinoma patients failed to demonstrate a statistically significant association with either gender or smoking status (data not shown). An analysis using CA-repeat length in the longer allele produced results similar to, but not as clear as, those obtained for the shorter allele (data not shown); hereafter, only the results for CA-repeats in the shorter allele are shown.

***EGFR* mRNA expression and CA-repeats in non-cancerous lung tissues.** *EGFR* mRNA levels in non-cancer tissues from 74 lung adenocarcinoma patients (excluding 39 non-informative cases),

doi: 10.1111/j.1349-7006.2008.00804.x

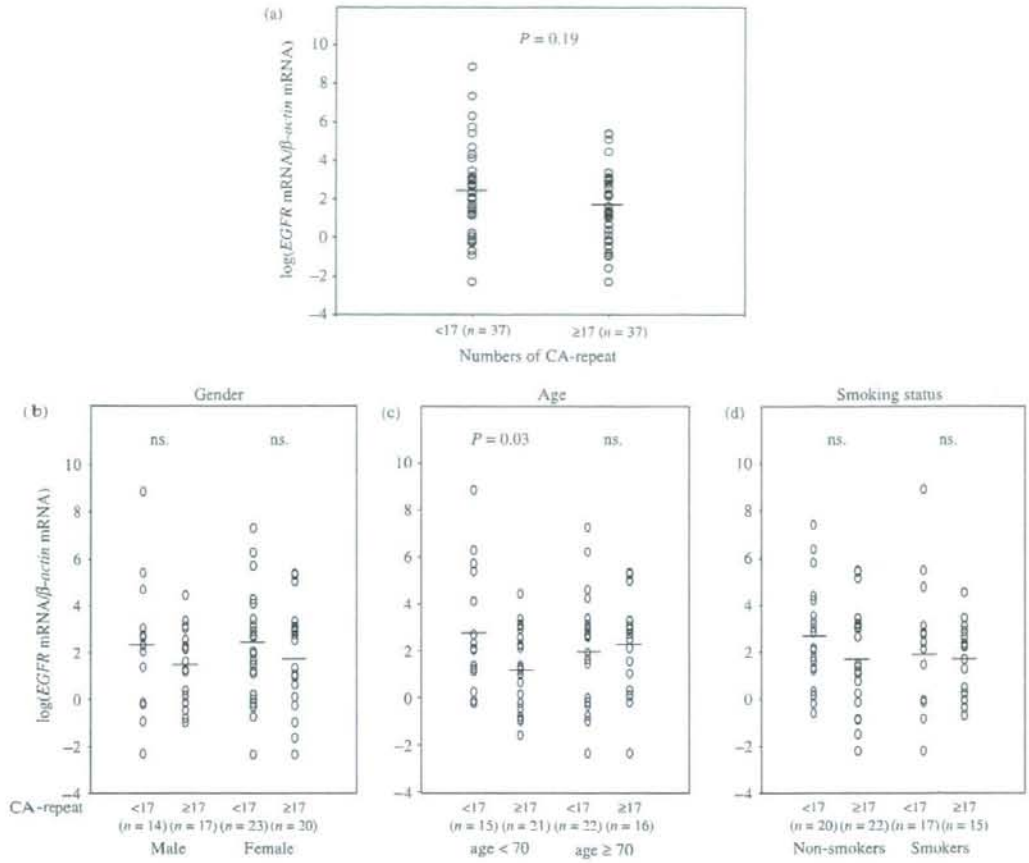

Fig. 2. Distribution of log(*epidermal growth factor receptor (EGFR)* mRNA/β-*actin* mRNA) in non-cancerous tissues among lung adenocarcinoma patients according to number of CA-repeats (<17 or ≥17) (a), gender (b), age (c), and smoking status (d). ns, not significant.

Table 2. Association between *EGFR* mutations and CA-repeat in 113 lung adenocarcinoma patients

	EGFR mutation			
	Present		Absent	*P*
	Deletion	Substitution		
Exon 18				
CA-repeat				
<17	0	1 (1.8%)	55 (98.2%)	ns†
≥17	0	2 (3.5%)	55 (96.5%)	
Exon 19				
CA-repeat				
<17	16 (28.6%)	0	40 (71.4%)	0.02
≥17	6 (10.5%)	0	51 (89.5%)	
Exon 21				
CA-repeat				
<17	0	10 (17.9%)	46 (82.1%)	ns
≥17	0	13 (22.8%)	44 (77.2%)	

†Not significant.
EGFR, epidermal growth factor receptor.

with adjustment for gender, age, and smoking status, did not differ significantly by CA-repeat length ($P = 0.19$, Fig. 2a). However, when patients were divided by age category according to the median age (70 years), increased levels of log(*EGFR* mRNA/β-*actin* mRNA) were associated with shorter CA-repeat length in the patients below age 70 ($P = 0.03$, Fig. 2c). Gender and smoking status may also influence the levels of log(*EGFR* mRNA/β-*actin* mRNA) (Fig. 2b,d, respectively). Therefore, we carried out a categorical regression analysis, with adjustment for the confounding variables (age, gender, and smoking status), and found that increased log(*EGFR* mRNA/β-*actin* mRNA) levels were associated with shorter CA-repeat length ($P = 0.02$, Table 3). As for cancer tissues, the median values of EGFR mRNA/β-actin mRNA were 10.7, and 9.8 in longer, and shorter CA-repeat length, respectively. No statistically significant association was found between CA-repeat length and *EGFR* mRNA levels in cancer tissues (data not shown).

Further examining the relationship between CA-repeat length and *EGFR* mutations at exon 19 according to whether log(*EGFR* mRNA/β-*actin* mRNA) was greater than or less than the median (2.10), we found a significantly increased frequency of *EGFR* mutations associated with shorter CA-repeat length only in the patients with higher *EGFR* mRNA expression ($P = 0.045$; Table 4).

Table 3. Categorical regression analysis of log(*EGFR* mRNA/*β-actin* mRNA) in 74 lung adenocarcinoma patients

Variables	β	*P*
Gender (male *versus* female)	−0.08	0.68
Age (<70 *versus* ≥70 years)	−0.12	0.31
Smoking status (non-smokers *versus* smokers)	−0.24	0.24
CA-repeat length (<17 *versus* ≥17)	−0.29	0.02

EGFR, epidermal growth factor receptor.

Logistic regression analysis of *EGFR* mutation frequency at exon 19, taking into account age, gender, and smoking status, also showed a significant contribution of shorter CA-repeat length in the patients with higher *EGFR* mRNA expression ($P = 0.03$, $\beta = -2.6$).

CA-repeat and growth of normal HBE cells. Normal HBE cells with CA 16/15 evidenced faster cell growth than those with CA 20/19 ($P = 0.004$, Fig. 3a). This is consistent with a shorter doubling time for HBE cells with CA 16/15 ($P = 0.006$, Fig. 3b). *EGFR* mRNA levels in HBE cells with CA 16/15 were significantly higher than those in HBE cells with CA 20/19 ($P = 0.017$, Fig. 3c). In addition, EGFR phosphorylation induced by EGF was enhanced in one clone with CA 16/15 and in another clone with CA 8/7, compared with that found in 3 clones with CA 20/19 (Fig. 4). These results suggest an inverse relationship between CA-repeat length and cell growth in HBE cells. Enhanced EGF/EGFR signaling was also found in HBE cells with shorter CA-repeats.

Discussion

EGFR mutations display different clinicopathological features according to the exons at which they occur. The majority of patients with mutations at exon 21 were female non-smokers who were diagnosed with adenocarcinomas showing bronchioloalveolar features, whereas patients with mutations at exon 19 included greater proportions of males and current or former smokers and a smaller proportion with bronchioloalveolar features.[10] *EGFR* mutations at exon 19 were small in-frame deletions, whereas those at exons 18 and 21 were base substitutions. Our findings that *EGFR* mutations were associated with gender, smoking status, and histological type, and occurred often in adenocarcinoma patients but rarely in squamous cell carcinoma patients, are consistent with previous reports.[7–10] Our finding

Table 4. *EGFR* mutations at exon 19 and CA-repeat length in relation to log(*EGFR* mRNA/*β-actin* mRNA) levels

		CA-repeat length		*P**
		<17	≥17	
log(*EGFR* mRNA/*β-actin* mRNA) < median†				
Mutations at exon19	Present	3	2	0.65
	Absent	14	17	
log(*EGFR* mRNA/*β-actin* mRNA) ≥ median				
Mutations at exon19	Present	7	1	0.045
	Absent	13	17	

*The exact *P*-value (two-sided) based on the Pearson χ^2 test.
†Median value of log(*EGFR* mRNA/*β-actin* mRNA) in non-cancerous tissues = 2.10.
EGFR, epidermal growth factor receptor.

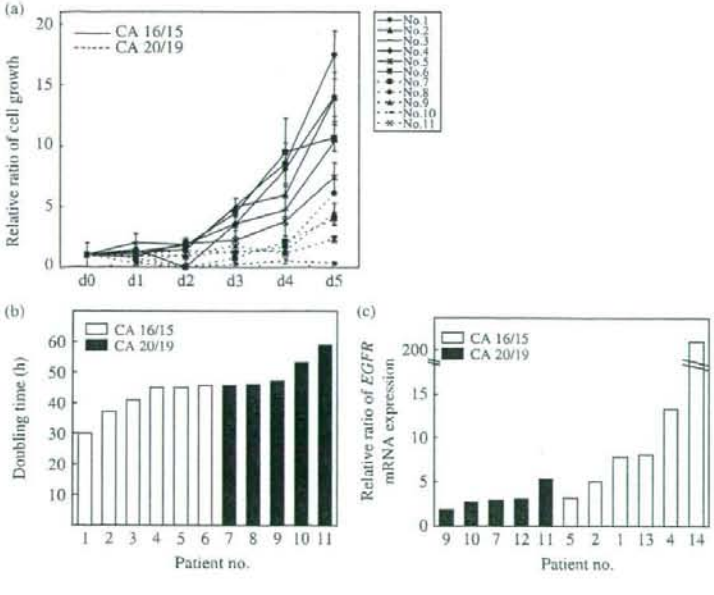

Fig. 3. Growth (a) and doubling time (b) of normal human bronchial epithelial (HBE) cells according to number of CA-repeats. Normal HBE cells were isolated from bronchial mucosal biopsy and subjected to experiments after three passages. Cells (4×10^4 cells/mL) were cultured in 0.5 mL medium, and the number of cells was determined by trypan blue staining after incubation for the indicated period. Relative cell numbers, which were divided by the cell number on day 0, are shown and the values are expressed as means ± SD of triplicate analyzes. (c) Levels of *epidermal growth factor receptor (EGFR)* mRNA in normal HBE cells as determined using real-time reverse transcription-polymerase chain reaction. The relative expression levels of *EGFR* mRNA were determined after correction for *β-actin* mRNA as a control gene. NS, not significant.

doi: 10.1111/j.1349-7006.2008.00804.x

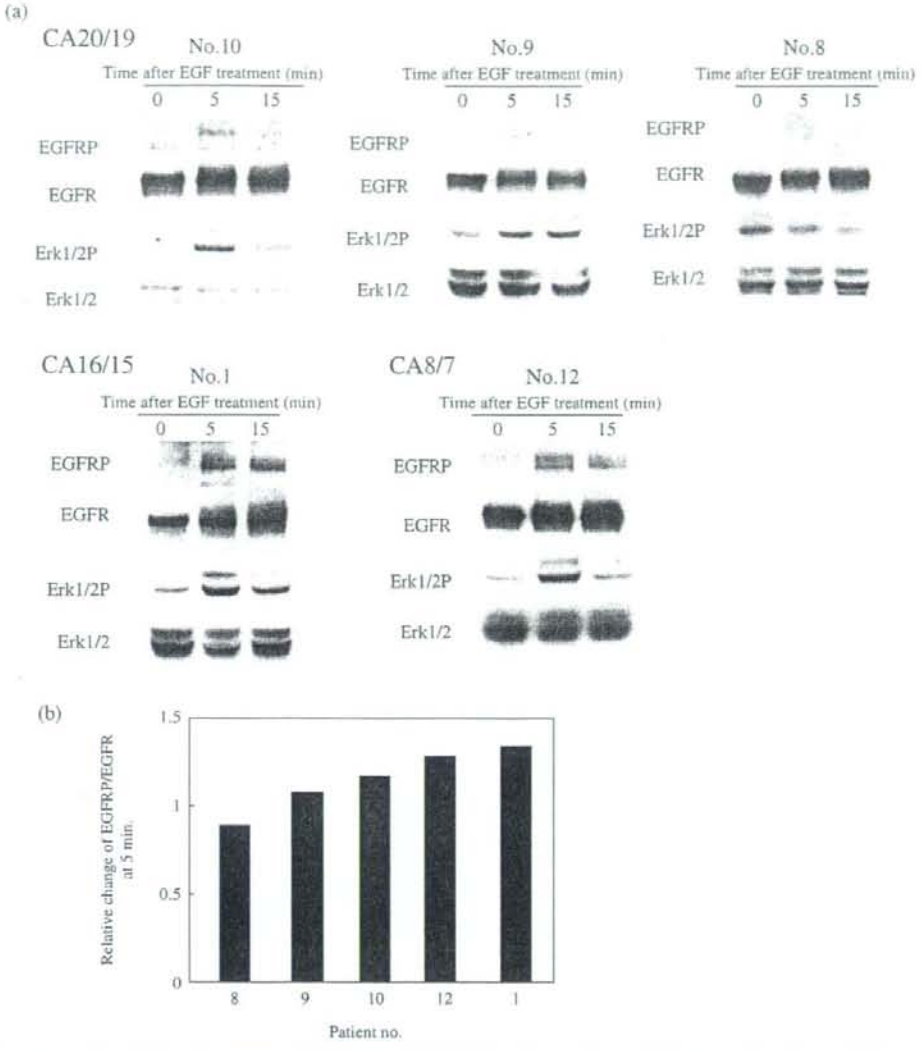

Fig. 4. *Epidermal growth factor receptor (EGFR)* phosphorylation induced by EGF was more apparent in the cells with CA-repeat lengths in the longer/shorter alleles of 16/15 and 8/7 compared to cells with CA-repeat lengths of 20/19. Normal human bronchial epithelial (HBE) cells were first incubated for 24 h in medium without EGF and bovine pituitary extract, then treated with 100 ng/mL EGF for the indicated periods. The numbers of CA-repeats were 20/19 in patients 10, 9, 8, 16/15 in patient 1, and 8/7 in patient 12. Western blot analysis was conducted on whole cell lysates (50 μg) (a). The relative ratio of EGFR phosphorylation was determined as the intensity of EGFR phosphorylation divided with that of EGFR (b).

that CA-repeat length at intron 1 of the *EGFR* gene displayed bimodal distributions in both the shorter and longer alleles is similar to a result reported in non-Chinese Asians.[23]

The results of the present study may be summarized as follows. First, *EGFR* mutations at exon 19 were closely associated with shorter CA-repeat lengths in the shorter allele, but this was not the case with *EGFR* mutations at exons 18 or 21. This implies that CA-repeat length is associated with deletion mutations, but not with substitution mutations. Second, the mechanism relating CA-repeat and *EGFR* mutations at exon 19 was elucidated by our finding that increased intrinsic *EGFR* mRNA expression in non-cancerous tissues was significantly associated with shorter CA-repeats. Third, the relationship between increased *EGFR* mutations and shorter CA-repeat length at exon 19 was apparent in lung adenocarcinoma patients with *EGFR* mRNA expression levels in the upper 50th percentile, but not in patients with lower *EGFR* expression levels. Fourth and lastly, our finding that increased cell growth and enhanced EGF/EGFR signaling occurred in HBE cells with shorter CA-repeat lengths and increased *EGFR* mRNA expression confirms the relationship between CA-repeat length and *EGFR* mRNA expression in the parallel studies mentioned above and provides a clue as to what cell phenotypes might arise from the shorter CA-repeat. Because EGF/EGFR signaling is enhanced in cells undergoing

rapid growth, and we found an inverse relationship between cell growth and CA-repeat length further related to *EGFR* mRNA levels. we conclude that *EGFR* mRNA levels are elevated in cells with shorter CA-repeat lengths, which results in enhanced EGF/EGFR signaling and therefore more rapid cell growth.

As for the mechanism linking CA-repeat length and *EGFR* mRNA expression, it has been postulated that the CA-repeat length at intron 1 influences DNA bendability and hence the binding of repressor protein.[29] However, we are puzzled as to how increased *EGFR* expression could be linked to increased *EGFR* mutations, specifically deletions. Our HBE study demonstrated that shorter CA-repeat lengths are associated with faster cell growth, which might result from increased *EGFR* expression and activation of EGF/EGFR signaling. Further study of the cell phenotypes related to shorter CA-repeat length in association with increased *EGFR* mutations is needed, though, because there are patients with *EGFR* mutations and shorter CA-repeat lengths with no evidence of elevated *EGFR* mRNA expression, which suggests that other mechanisms may be involved.

It has been reported recently that EGFR is also involved in DNA repair. EGFR binds to DNA-dependent protein kinase (DNA-PK) complex and induces the translocation of DNA-PK complex into the nucleus.[30,31] Mutated forms of EGFR abrogate ionizing radiation-induced nuclear EGFR translocation or binding to DNA-PK catalytic subunit (DNA-PKcs), resulting in inhibition of DNA-PK activity.[32] DNA-PK is involved in non-homologous end joining, one of the most important DNA repair systems in mammalians.[33] Because defects in the DNA-PK complex, Ku proteins, and DNA-PKcs enhance tumorigenecity in transgenic mice,[34–36] it is possible that *EGFR* mutations are causally related to cancer development; in fact, transgenic mice carrying the *EGFR* mutations $EGFR^{L858R}$ and $EGFR^{\Delta L747-S752}$ developed adenocarcinomas of the lung.[12] Taken together, these findings lead us to postulate that shorter CA-repeat lengths might enhance intrinsic *EGFR* mRNA expression through altered bendability of the gene, and up-regulated EGFR might cause enhanced cell growth and attenuated DNA repair capacity – specifically against deletions – through physical interaction with DNA-PK complex, resulting in increased *EGFR* mutations in normal lung epithelial cells.

Here we report that an association between *EGFR* mRNA expression and CA-repeat length was found only in non-cancerous tissues. That *EGFR* mRNA expression in cancer tissues was not highly correlated with that in corresponding non-cancerous tissues (correlation coefficient 0.34) is consistent with our finding that cancer tissues did not evidence an association between *EGFR* expression and CA-repeat length. This may be due to the fact that intrinsic *EGFR* expression is altered in cancer cells by various factors including mutations as well as allelic imbalance of *EGFR*, which has also been reported to be associated with CA-repeat length.[25]

As for interethnic differences in CA-repeat length, lengths of less than 17 in Japanese are less frequent than in Caucasians.[23] However, the frequency of *EGFR* mutations is higher in Japanese than in other ethnic groups (primarily Caucasians): 30–50% versus 5–20%.[37–39] Mutations of other genes in lung cancer, specifically *KRAS* and *TP53*, are more frequent in Caucasians than in Japanese. *KRAS* and *TP53* mutations are known to be caused by smoking, and in particular the G:C-to-T:A transversion in *TP53* is generally interpreted as a mutagen footprint.[40] However, *KRAS* and *TP53* mutations occur at relatively high rates even among non-smokers in Japanese (6% and 30%, respectively[7]) and at even higher rates in Caucasians (10% and 47.5%, respectively[40]). *KRAS* and *EGFR* mutations are mutually exclusive, and either type of mutation alone is thought to be sufficient for lung carcinogenesis. These factors relative to mutations in other genes may contribute to interethnic differences in *EGFR* mutation rates as well as different genetic backgrounds.

In future research, we plan to investigate whether the CA-repeat polymorphism can serve as an alternative predictive marker for EGFR tyrosine kinase inhibitor response. We also intend to expand our research on the relationship between CA-repeat and *EGFR* mutations in lung carcinogenesis.

Acknowledgments

This work was supported by grants from the Ministry of Education, Science, Sports and Culture (19591116, Japan), and the Smoking Research Foundation.

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Stroke

JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Cigarette Smoking, Systolic Blood Pressure, and Cardiovascular Diseases in the Asia-Pacific Region

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Stroke 2008;39;1694-1702; originally published online Mar 6, 2008;
DOI: 10.1161/STROKEAHA.107.496752
Stroke is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0039-2499. Online ISSN: 1524-4628

Cigarette Smoking, Systolic Blood Pressure, and Cardiovascular Diseases in the Asia-Pacific Region

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Background and Purpose—Smoking and increased levels of blood pressure (BP) substantially increase the risk of cardiovascular diseases (CVD). If these 2 risk factors have a synergistic impact on cardiovascular events, lowering BP and quitting smoking will contribute more to reducing CVD than would be expected from ignoring their interaction.

Methods—Individual participant data were combined from 41 cohorts, involving 563 144 participants (82% Asian). During a median of 6.8 years follow-up, 4344 coronary heart disease (CHD) and 5906 stroke events were recorded. Repeat measures of systolic blood pressure (SBP) were used to adjust for regression dilution bias. Hazard ratios (HRs) and 95% confidence intervals (CIs) for SBP by cigarette smoking status were estimated from Cox proportional hazard models adjusted for age and stratified by study and sex.

Results—Data suggested a log-linear relationship between SBP and all subtypes of CVD. The HRs relating SBP to both CHD and ischemic stroke were broadly similar irrespective of smoking status ($P \geq 0.1$). For hemorrhagic stroke (intracerebral hemorrhage), the HRs (95% CIs) for an additional 10 mm Hg increment in SBP were 1.81 (1.73 to 1.90) for present smokers and 1.66 (1.59 to 1.73) for nonsmokers ($P=0.003$). For every subtype of cardiovascular events, similar results were found for analyses involving only fatal events.

Conclusions—Smoking exacerbated the impact of SBP on the risk of hemorrhagic stroke. Although quitting smoking and lowering BP are both crucial for prevention of CVD, combining the 2 could be expected to have extra beneficial effect on preventing hemorrhagic stroke. **(*Stroke*. 2008;39:1694-1702.)**

Key Words: smoking ■ blood pressure ■ cardiovascular diseases ■ coronary heart disease ■ stroke

Nonoptimal levels of blood pressure (BP) and smoking are the first and second most common causes of death in the world, and, together, these 2 risk factors account for more than 20% of the global burden of premature death.[1,2] In particular, increased BP[3–7] and smoking[7–11] are major risk factors for cardiovascular diseases (CVD), including coronary heart disease (CHD) and stroke. Previous studies have indicated that smoking and increased BP interact to increase markers of cardiovascular risk, including levels of plasma fibrinogen[12] and carotid intima-media thickness.[13] Hence, a combination of raised BP and smoking may have a synergistic impact on cardiovascular events, especially those caused by atherosclerosis and thrombosis.[14] If such an interaction exists, multifactorial interventions aimed at both lowering BP and quitting smoking will contribute more to reducing CVD than expected from past data where their interaction has not been quantified.

Several epidemiological studies have examined the combined effects of nonoptimal levels of BP and smoking on cardiovascular events.[11,15–23] Some studies, at least partially, observed a synergistic effect between BP and smoking status for the risk of CVD,[15] CHD,[16–18] and stroke (predominately ischemic),[11,16,19,20] whereas other studies did not observe any such effect.[21,22] The majority of these studies were based on small datasets and crude classifications of BP and smoking status, and few examined the possible interaction effect between BP and smoking status for each subtype of CVD. For hemorrhagic stroke, only 1 case-control study[23] examined the interaction between BP and smoking status; it reported that interaction was present. Overall, however, the question as to

Received June 14, 2007; final revision received September 13, 2007; accepted October 26, 2007.

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Stroke is available at http://stroke.ahajournals.org DOI: 10.1161/STROKEAHA.107.496752

whether such an interaction exists, and the nature of this interaction (synergistic or otherwise), remains unresolved. The aim of the present study was to examine this issue using data from the Asia Pacific Cohort Studies Collaboration (APCSC); an individual participant data overview of prospective cohort studies conducted in the Asia-Pacific region. The large size of the dataset provides an ideal opportunity to explore the joint associations of risk factors with cardiovascular events. In particular, the large numbers of both hemorrhagic and ischemic stroke events makes it possible to measure the risk for each subtype of stroke reliably. Additionally, APCSC provides a unique opportunity to compare the association of risk factors with cardiovascular events between Asian populations and the "Western" populations of Australia and New Zealand.

Methods

Participating Studies

Details of APCSC are described elsewhere.[24,25] Briefly, APCSC is an overview of preexisting cohort studies in the Asia-Pacific region which had at least 5000 person-years of follow-up and recorded age, sex, and BP at baseline, and vital status at the end of the follow-up. Studies were excluded from APCSC if enrolment was dependent on having a particular condition or risk factor. Additionally, for analyses in this report, only persons aged ≥20 years at study entry with information on both BP and smoking status were included.

Measurement of Baseline Variables

In most studies, BP was measured at rest in the seated position using a standard mercury sphygmomanometer. Cigarette smoking habit was self-reported at study baseline. All studies included here recorded present smoking status (present smoker or not). Some studies additionally recorded whether individuals were present, former, or never smokers, and some recorded cigarettes per day for smokers. Because most studies, including APCSC, have demonstrated that the association between systolic blood pressure (SBP) and cardiovascular events is stronger than that of other BP indices in most age and gender groups,[26,27] we analyzed data on SBP in this report. Cohorts were classified as Asian if the participants were recruited from mainland China, Hong Kong, Japan, Korea, Singapore, Taiwan, or Thailand and as ANZ if the participants were from Australia or New Zealand. This classification largely represented a split by ethnicity into Asians and Whites.

Outcomes

All studies reported deaths by underlying cause; a subset of studies also reported nonfatal cardiovascular events. Outcomes were classified according to the Ninth Revision of the International Classification of Diseases (ICD-9). Outcomes in this report, including fatal and nonfatal events, were CHD (ICD-9: 410 to 414) and stroke (430 to 438), divided into hemorrhagic stroke (intracerebral hemorrhage; 431.0 to 432.9), ischemic stroke (433.0 to 434.9), and other strokes. Because most studies identified events using record linkage, verification of pathological types of stroke was not routinely reported. All data provided to the Secretariat were checked for completeness and consistency and recoded, when necessary, to maximize comparability across cohorts. Summary reports were referred back to principal investigators of each collaborating study for review and confirmation.

Statistical Methods

Cox proportional hazard regression models adjusted by age and stratified by study and sex[28] were used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for SBP by smoking status (nonsmokers, including former smokers, and present smokers). To determine the associations between "usual" level of SBP and the outcomes of interest, estimates were adjusted for regression dilution bias.[3,29] Repeat measurements of SBP on up to 7 occasions, between 2 and 20 years after the baseline measurement, were obtained from 16 studies for a total of 67 210 participants. These repeat measures were used to estimate a regression dilution attenuation coefficient for SBP (1.9), using a linear mixed regression model that accounted for the heterogeneity of variance between studies and within-subject correlation.[30] Log-linearity of the associations between SBP and each subtype of cardiovascular event was explored by categorical analyses in which participants were classified into 4 groups according to levels of baseline SBP (<130, 130 to 144, 145 to 159, and ≥160 mm Hg) chosen so as to have approximately equal numbers of all cardiovascular events across the groups. Corresponding 95% CIs were calculated by the "floating absolute risk method."[29] HRs and 95% CIs were also derived for a 10 mm Hg increase in the level of SBP. The interaction effect between SBP and smoking status was assessed using likelihood ratio tests comparing the models with main effects only with the models that included the interaction term.[29] In addition to analyses of the overall APCSC, predefined subgroup analyses were performed by sex, region (Asia and ANZ), and age at risk (<65 and ≥65 years).[24]

Further analyses were conducted on subsamples of the total population which had more detailed information on smoking status. In one of the subsamples, participants were classified as "present" if they smoked currently, "never smokers" if they had never smoked, and "former smokers" if they had smoked but reported having already quit at study baseline. HRs for a 10-mm Hg increase in the level of SBP were estimated for each group by this smoking status and compared using similar methods to the main analyses. Similarly, dose-response analyses were done on the subset where both the mean number of cigarettes smoked per day and never smoking were recorded, comparing never smokers with <20 and ≥20 cigarettes per day for present smokers. Groups of <20 and ≥20 were chosen to provide an approximately equal partition; 20 cigarettes corresponds to 1 standard pack.

Results

Characteristics of the Study Population

Information on SBP and smoking status was available from 41 cohorts (93% of all studies in APCSC); 32 from Asia (Table 1). Overall, 563 144 participants were included in the analysis (82% Asians; 35% female) with a mean age of 47 years. Over one-third (37%) of study participants were classified as present smokers at baseline, but the prevalence of smoking differed by sex and region: in Asia, 59% of men and 5% of women were present smokers versus 20% and 14%, respectively, in ANZ. In Asia, mean age and SBP were similar between smokers and nonsmokers (45 years versus 45 years and 122 mm Hg versus 121 mm Hg), but in ANZ, present smokers were both younger and had a lower SBP than nonsmokers: 48 years versus 54 years and 133 mm Hg versus 138 mm Hg, respectively. These mean values of age and SBP were weighted, rather than crude, averages across studies.

Information on former smoking status was available from 34 cohorts (24 in Asia). In these, 63 941 (13%) of participants were former smokers, 261 319 (51%) were never smokers, and 187 416 (37%) were present smokers. In Asian cohorts, 15% of men and 22% of women who had ever smoked had quit, compared to 68% and 59%, respectively, in ANZ. Of these 34 cohorts, 24 also recorded information on the average number of cigarettes smoked per day. Among the 97 540 present smokers in these cohorts, 44% consumed 20 cigarettes or more per day: in Asia, 44% for men and 21% for women, versus 52% and 43%, respectively, in ANZ.

Table 1. Study Population Characteristics by Smoking Status at Baseline

	Nonsmokers				Current Smokers			
Study Name	n	Age (years) mean (SD)	SBP (mm Hg) mean (SD)	Female (%)	n	Age (years) mean (SD)	SBP (mm Hg) mean (SD)	Female (%)
Akabane	1321	55 (8)	125 (19)	77	513	53 (7)	124 (19)	2
Anzhen	5992	54 (13)	129 (24)	69	2386	53 (12)	130 (22)	20
Anzhen02	3287	47 (8)	122 (18)	64	864	46 (8)	122 (17)	1
Beijing aging	1472	70 (9)	143 (25)	62	620	69 (8)	137 (25)	24
Capital Iron Steel Company	1367	45 (8)	125 (19)	0	3775	45 (8)	123 (19)	0
CISCH	1576	44 (7)	117 (17)	69	591	45 (8)	122 (16)	2
Civil service workers	5739	47 (5)	125 (18)	47	3501	47 (5)	126 (18)	10
CVDFACTS	4455	47 (15)	118 (19)	70	1274	48 (16)	119 (18)	4
East Beijing	806	45 (15)	125 (23)	64	322	41 (15)	124 (21)	20
EGAT	1980	43 (5)	121 (17)	38	1514	43 (5)	121 (16)	3
Fangshan	1591	47 (10)	136 (26)	86	1028	48 (10)	135 (25)	36
Guangzhou occupational	87 400	41 (6)	115 (15)	41	79 295	42 (7)	116 (14)	1
Hisayama	918	57 (12)	135 (26)	82	683	55 (10)	135 (26)	22
Hong Kong	2428	79 (7)	150 (25)	63	555	77 (6)	148 (24)	33
Kinmen	1824	63 (10)	138 (23)	64	721	64 (9)	136 (21)	9
KMIC	98 631	44 (7)	121 (14)	54	61 611	45 (7)	125 (14)	0
Konan	857	52 (16)	130 (20)	75	369	51 (16)	130 (18)	9
Miyama	756	61 (10)	134 (22)	73	317	60 (9)	130 (22)	13
Ohasama	1793	60 (11)	127 (17)	78	447	58 (12)	132 (18)	7
Saitama	2588	54 (12)	135 (20)	80	1027	55 (12)	136 (19)	17
Seven cities cohorts	7019	54 (12)	130 (25)	70	3792	54 (12)	129 (23)	26
Shanghai factory workers	5198	47 (7)	124 (21)	51	4149	50 (7)	126 (23)	5
Shibata	1573	57 (11)	130 (21)	82	777	57 (11)	133 (20)	8
Shigaraki town	2657	58 (14)	132 (19)	77	1073	56 (14)	132 (20)	16
Shirakawa	3023	48 (12)	127 (22)	79	1617	48 (12)	126 (21)	8
Singapore heart	1807	40 (13)	124 (22)	61	514	41 (14)	122 (18)	7
Singapore NHS92	2699	39 (12)	119 (19)	62	606	39 (12)	118 (17)	8
Six cohorts	10 465	44 (7)	119 (18)	76	8922	45 (7)	119 (17)	12
Tanno/Soubetsu	1214	51 (7)	134 (20)	78	764	51 (7)	132 (21)	14
Tianjin	4586	56 (13)	139 (28)	64	4749	54 (11)	134 (25)	39
Xi'an	1020	44 (6)	126 (21)	49	675	45 (6)	125 (20)	10
Yunnan	2138	58 (10)	126 (22)	9	4443	55 (9)	123 (21)	0
Total Asia	270 180	45 (10)	121 (18)	53	193 494	45 (9)	122 (17)	4
ALSA	1486	78 (6)	148 (22)	48	124	76 (6)	148 (26)	48
ANHF	7043	44 (14)	126 (18)	53	2234	41 (13)	125 (18)	45
Busselton	5155	45 (17)	138 (25)	59	2634	44 (16)	137 (24)	37
Canberra	728	77 (5)	145 (21)	46	93	76 (5)	147 (22)	39
Fletcher challenge	7899	46 (15)	127 (17)	30	2427	40 (13)	124 (15)	22
Melbourne	36 630	55 (9)	138 (20)	60	4655	53 (8)	135 (19)	47
Newcastle	4567	52 (11)	133 (20)	53	1362	50 (10)	131 (20)	40
Perth	7625	46 (13)	130 (20)	51	2605	43 (13)	129 (19)	40
WAAAAS	10 870	72 (4)	157 (21)	0	1333	71 (4)	157 (22)	0
Total ANZ	82 003	54 (14)	138 (22)	47	17 467	48 (15)	133 (22)	37
Total	352 183	47 (12)	125 (20)	52	210 961	45 (9)	123 (18)	7

SD indicates standard deviation; SBP, systolic blood pressure; ANZ, Australia and New Zealand; ALSA, Australian Longitudinal Study of Aging; ANHF, Australian National Heart Foundation; CISCH, Capital Iron and Steel Company Hospital; EGAT, Electricity Generating Authority of Thailand; KMIC, Korean Medical Insurance Corporation; NHS92, National Health Study 1992; WAAAAS, Western Australian AAA Screenees.

Cardiovascular Outcomes

In total, there were 3 907 543 person-years of follow-up; the median follow-up was 6.8 years (6.8 years for present smokers and 6.7 years for nonsmokers) but, for both present smokers and nonsmokers, it was shorter in Asia (6.8 years versus 6.0 years) than in ANZ (8.3 years versus 8.2 years; Table 2). In addition to information on fatal events available from all cohorts, data on nonfatal CHD events were available from 14 studies and on nonfatal strokes from 12 studies. During follow-up, 4344 CHD (1569 in Asia) and 5906 stroke (4218 in Asia) fatal and nonfatal events were recorded: 76% (n=3282) of CHD events were fatal. Over 80% of CHD events were myocardial infarction. Of all stroke events, 2001 (1550 in Asia) were classified as ischemic and 1645 (1441 in Asia) as hemorrhagic: 30% (n=608) of ischemic stroke and 73% (n=1207) of stroke events were fatal. Diagnosis of ischemic or hemorrhagic stroke was documented by CT/MRI/autopsy investigations in 56% of fatal and 65% of nonfatal, strokes. The percentage of CHD among all CVD (CHD plus stroke) was similar between smokers and nonsmokers (40% versus 44%): these percentages in ANZ (61% versus 64%) were more than double those in Asia (29% versus 25%). The percentage of hemorrhagic strokes among all strokes was similar between smokers and nonsmokers (30% versus 26%): these percentages were higher in Asia (34% versus 34%) than in ANZ (13% versus 12%).

The Association Between SBP and CHD by Smoking Status

The HR for CHD increased log-linearly with higher levels of SBP in both smokers and nonsmokers (Figure 1A). The HRs (95% CIs) comparing the top to the bottom group of SBP were 2.27 (2.05 to 2.52) for present smokers and 2.20 (2.05 to 2.36) for nonsmokers. The HR for a 10-mm Hg increase in SBP level was also similar for present smokers and nonsmokers (Figure 2): 1.29 (1.24 to 1.34) and 1.24 (1.21 to 1.28), respectively (probability value for interaction=0.14). The coronary HRs for present smokers and nonsmokers were similar in all sex, age, and region subgroups. Similar results (not shown) were found for analyses involving fatal events only.

In the subsample of studies for which information on former smokers was available, the HRs for CHD associated with a 10-mm Hg increase in SBP were similar for present smokers and never smokers. However, the HR was lower in former smokers than in present or never smokers: 1.28 (1.22 to 1.33) for present smokers, 1.14 (1.09 to 1.20) for former smokers, and 1.30 (1.25 to 1.35) for never smokers (probability value for interaction=0.0001). In the subsample of studies with information on cigarette consumption, the HRs for CHD tended to increase with increasing consumption of cigarettes: 1.27 (1.21 to 1.32) for never smokers, 1.30 (1.19 to 1.43) for <20 cigarettes per day, and 1.41 (1.28 to 1.54) for ≥20 cigarettes per day (probability value for interaction=0.11).

The Association Between SBP and Ischemic Stroke by Smoking Status

Similar to CHD, there was no evidence of an interaction between BP and smoking for risk of ischemic stroke: the HR for ischemic stroke increased log-linearly with higher levels of SBP in both present smokers and nonsmokers (Figure 1B). The HRs (95% CIs) comparing the highest with the lowest group of SBP were 3.71 (3.22 to 4.27) for present smokers and 3.82 (3.43 to 4.26) for nonsmokers. The HR for a 10-mm Hg increase in SBP level was similar for present smokers and nonsmokers in all subgroups (Figure 2). Overall HRs (95% CIs) were 1.50 (1.43 to 1.57) for present smokers and 1.47 (1.41 to 1.53) for nonsmokers (probability value for interaction=0.53). Similar results (not shown) were found for analyses involving fatal events only.

In the subsample with information on former smokers, the HR for a 10-mm Hg increase in SBP was similar for present smokers, former smokers, and never smokers: 1.44 (1.36 to 1.52), 1.41 (1.29 to 1.53), and 1.41 (1.34 to 1.49), respectively (probability value for interaction=0.86). Among those participants with information on cigarettes per day there was marginally nonsignificant evidence of an increasing effect of SBP with increasing cigarette consumption. The HRs were 1.30 (1.20 to 1.41) for never smokers, 1.47 (1.26 to 1.70) for <20 cigarettes per day, and 1.62 (1.34 to 1.97) for ≥20 cigarettes per day (probability value for interaction=0.06).

The Association Between SBP and Hemorrhagic Stroke by Smoking Status

The HR for hemorrhagic stroke increased with higher levels of SBP in both present smokers and nonsmokers (Figure 1C). There was evidence to support a synergistic effect of smoking on the association between SBP and hemorrhagic stroke risk: the HRs (95% CIs) for hemorrhagic stroke comparing the group with the highest to that with the lowest SBP values were 9.32 (8.15 to 10.67) for present smokers and 7.05 (6.27 to 7.92) for nonsmokers. The excess risk of hemorrhagic stroke associated with a 10-mm Hg higher SBP level increased in present smokers compared with nonsmokers by 15 percentage points (ie, 81% versus 66%) (Figure 2): 1.81 (1.73 to 1.90) versus 1.66 (1.59 to 1.73); probability value for interaction=0.003. Subgroup analysis found indications of this synergistic effect in most subgroups, although it was statistically significant only for men ($P=0.01$), in Asian study centers ($P=0.05$), and individuals aged 65 years or over ($P=0.008$) (Figure 2). Restricting the analysis to fatal hemorrhagic events resulted in a similar pattern: HR (95% CI) for a 10-mm Hg increase in SBP was 1.82 (1.72 to 1.92) for present smokers and 1.67 (1.59 to 1.75) for nonsmokers (probability value for interaction=0.01).

The HR for a 10-mm Hg increase in SBP was higher in present smokers than in former smokers and never smokers: 1.87 (1.77 to 1.97) versus 1.55 (1.40 to 1.71) and 1.68 (1.58 to 1.78), respectively (probability value for interaction=0.0008). In the subsample with information on cigarettes per day, the HRs increased with higher dose of smoking: 1.60 (1.47 to 1.75) for never smokers, 1.85 (1.65 to 2.08) for <20 cigarettes per day, and 1.95 (1.72 to 2.22) for ≥20 cigarettes per day (probability value for interaction=0.01).

A sensitivity analysis using only data from participants (n=126 956) in which information on the use of antihypertensive medication status at study baseline was available indicated that further adjustment for use of antihypertensive

Table 2. Fatal and Nonfatal Cardiovascular Events by Smoking Status

	Nonsmokers					Current Smokers				
			Stroke					Stroke		
Study Name	Median FUP	CHD	Isch	Hem	Others	Median FUP	CHD	Isch	Hem	Others
Akabane	11.0	15	9	5	11	11.0	13	7		6
Anzhen	4.3	50	74		7	4.3	15	32	20	3
Anzhen02	3.0		11	43		3.0	1	3	1	1
Beijing aging	4.8				61	4.8				25
Capital Iron Steel Company	12.5	13	15	20		12.5	70	77	45	9
CISCH	3.3	9			6	3.3	5			3
Civil service workers	6.7					6.7	1		1	1
CVDFACTS	6.1	10	6	5	10	5.8	3	1	3	4
East Beijing	16.0	12	10	8	2	17.4	8	4	3	1
EGAT	11.4	9			8	11.4	24			8
Fangshan	3.6	2	15	6	4	3.6	3	5	2	2
Guangzhou occupational	7.3	60		68	37	7.2	106		99	58
Hisayama	25.1	40	129	29	19	22.6	49	101	39	11
Hong Kong	2.5	73	5	14	41	2.5	13	1	2	10
Kinmen	2.9	6			8	2.9	4			6
KMIC	4.0	107	187	161	150	4.0	171	245	164	147
Konan	6.4		6	2	2	6.4	2	1	1	
Miyama	6.6	1	2		2	6.6	1	4	1	1
Ohasama	4.1	2	21	9	4	4.1	5	16	2	2
Saitama	11.0	14	19	9	10	10.0	10	8	6	3
Seven cities cohorts	2.7	51	66	109	6	2.7	33	51	73	2
Shanghai factory workers	14.0	33			114	14.0	53			141
Shibata	20.0	40	46	23	62	20.0	27	31	13	34
Shigaraki town	4.4	2	2	2	1	4.4	1	2		6
Shirakawa	17.5	29	18	20	12	17.5	36	21	11	5
Singapore heart	14.7	40	16	6	37	14.2	26	6	1	9
Singapore NHS92	6.2	22	11	1	19	6.2	11	3	3	8
Six cohorts	9.0	6	33	50	7	8.3	41	71	41	6
Tanno/Soubetsu	16.4	8	7	7	5	16.4	16	3	9	2
Tianjin	6.1	65	58	97	43	6.1	49	64	90	22
Xi'an	19.7	12	8	17	2	19.7	23	7	7	
Yunnan	4.5	7	5	42		4.5	11	7	51	1
Total Asia	6.0	738	779	753	690	6.8	831	771	688	537
ALSA	4.7	77	7	8	34	3.3	4			3
ANHF	8.4	55	1		10	8.3	22	1		5
Busselton	26.5	767	153	57	407	26.5	480	85	40	207
Canberra	9.6	106	5	4	23	8.4	14	1	1	4
Fletcher challenge	5.7	202	56	7	101	5.8	71	11	2	17
Melbourne	8.5	262	10	28	43	8.7	61	1	7	11
Newcastle	8.5	78	3	6	15	9.4	59		3	7
Perth	14.4	127	3	7	29	14.4	68	1	3	20
WAAAAS	3.2	285	98	29	86	3.2	37	15	2	11
Total ANZ	8.2	1959	336	146	748	8.3	816	115	58	285
Total	6.7	2697	1115	899	1438	6.8	1647	886	746	822

FUP indicates follow-up (years); CHD, coronary heart disease; Isch, ischemic; Hem, hemorrhagic; ANZ, Australia and New Zealand; ALSA, Australian Longitudinal Study of Aging; ANHF, Australian National Heart Foundation; CISCH, Capital Iron and Steel Company Hospital; EGAT, Electricity Generating Authority of Thailand; KMIC, Korean Medical Insurance Corporation; NHS92, National Health Study 1992; WAAAAS, Western Australian AAA Screenees; Blanks indicate that the event was not reported for that study.

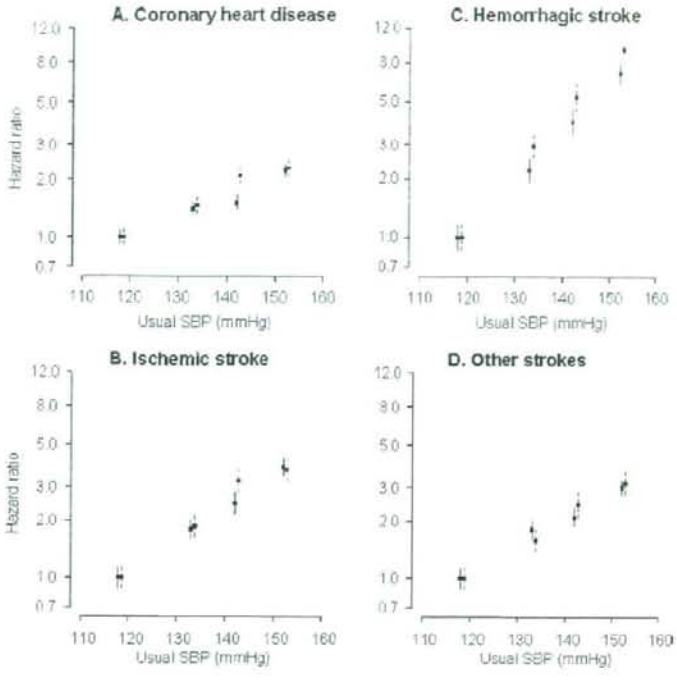

Figure 1. Associations between usual systolic blood pressure (SBP) and overall events by smoking status for: (A) coronary heart disease, (B) ischemic stroke, (C) hemorrhagic stroke, and (D) other strokes. The hazard ratio (95% confidence interval) for the lowest group of SBP is fixed at 1.0, separately for present smokers and nonsmokers. Analyses are adjusted by age and stratified by study and sex. The dashed (right) and continuous (left) lines represent present smokers and nonsmokers, respectively. (Probability values for log-linearity <0.0001 for all.)

medication did not attenuate the difference in risk estimates between present smokers and nonsmokers. The HR (95% CI) for a 10-mm Hg increase in SBP was 1.42 (1.24 to 1.63) for present smokers and 1.23 (1.11 to 1.36) for nonsmokers, after age adjustment (probability value for interaction=0.09), and 1.39 (1.21 to 1.60) and 1.20 (1.09 to 1.33), respectively, after age and use of antihypertensive medication adjustment (probability value for interaction=0.08).

The Association Between SBP and Other Strokes by Smoking Status

For completeness, Figure 1D shows the categorical analyses for other strokes. As with ischemic and hemorrhagic strokes, the HR increased with higher levels of SBP in both present smokers and nonsmokers. The HRs (95% CIs) comparing the highest with the lowest group of SBP were 3.17 (2.76 to 3.64) for present smokers and 3.01 (2.76 to 3.64) for nonsmokers. The HRs (95% CIs) for a 10-mm Hg increment in SBP level were 1.40 (1.33 to 1.47) in present smokers and 1.36 (1.31 to 1.41) in nonsmokers (probability value for interaction=0.33).

Discussion

The present study demonstrates a log-linear relationship of SBP with every subtype of CVD, for both smokers and nonsmokers, with no evidence of a threshold effect down to usual levels of SBP of 115 mm Hg. For hemorrhagic stroke, there was evidence that SBP and smoking have a synergistic effect such that smoking increases the excess risk associated with a 10-mm Hg increment in SBP by about 15 percentage points. Our data suggest that this interaction may be specific to men and older participants, but is unlikely to be specific to region because of the marginal differences between smokers and nonsmokers in both Asia and ANZ apparent from Figure 2. By comparison, the excess relative risk associated with increments in SBP for both CHD and ischemic stroke was broadly similar for smokers and nonsmokers.

The prevailing cause of CHD and ischemic stroke is occlusion of the coronary and cerebral arteries due to atherosclerosis and thrombosis.[14] Some previous reports suggest that nonoptimal levels of BP combined with smoking may promote atherothrombogenesis.[12,13] Kiyohara and colleagues[16] observed an interaction effect between BP and smoking status for CHD in women but not in men, and 1 study[17] observed such an effect in women. Meanwhile, 1 study[18] observed such an effect in men. In a case-control study, Ohgren and colleagues[19] reported an interaction effect between BP and smoking status for all strokes (78% of which were ischemic). Two Japanese studies[11,16] observed such a potentiation for ischemic stroke among men (but not women[16]), as did the British Regional Heart Study,[20] in which the majority of strokes would be expected to be ischemic in origin. By contrast, 2 studies[21,22] in populations where ischemic stroke predominates did not observe such a potentiation for all strokes. These null findings are consistent with our results based on the simple assessment of present smoking status (ie, present/nonsmokers, and present/former/never smokers), suggesting that smoking does not exacerbate the association between SBP and the risk of CHD and ischemic stroke. Furthermore, as most of the previous studies used a relatively crude classification of smoking and hypertensive status, previous positive findings of an interaction may have been attributable to chance alone. There was however some sug-

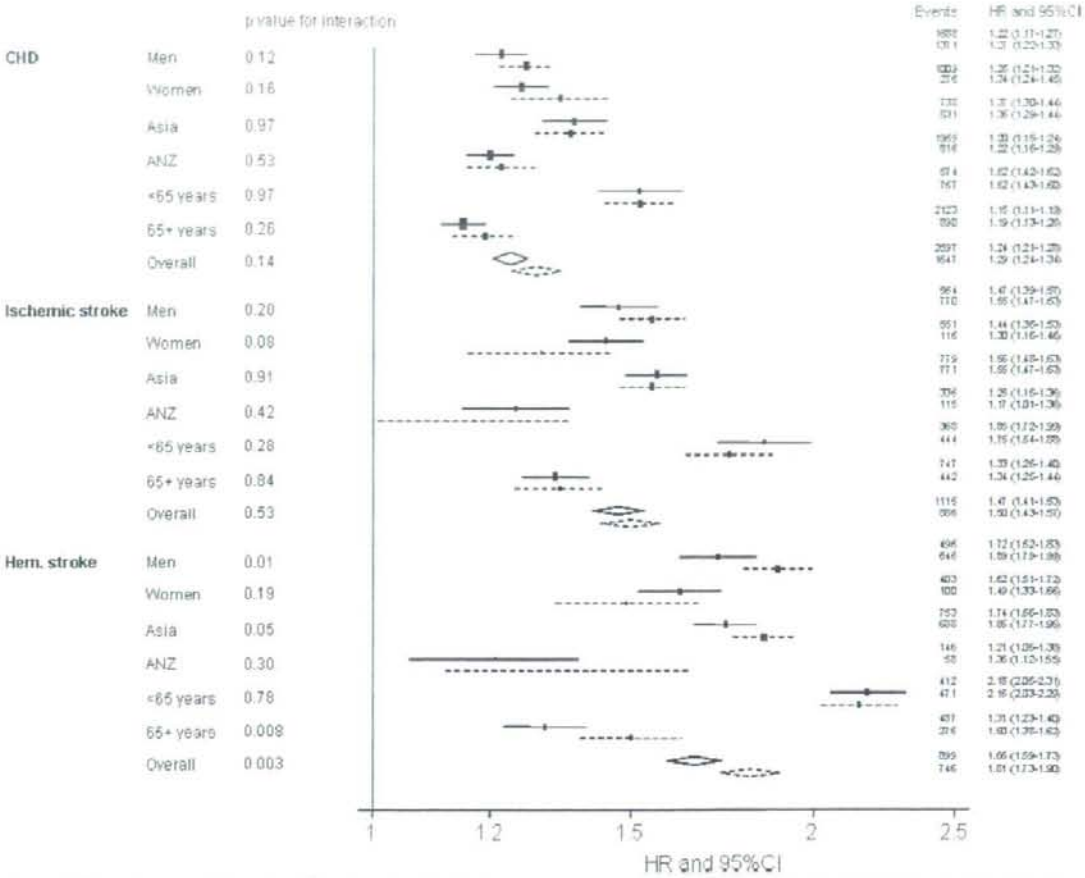

Figure 2. Hazard ratios (HRs) associated with a 10-mm Hg increase in usual systolic blood pressure for coronary heart disease (CHD), ischemic stroke, and hemorrhagic (Hem) stroke, in present smokers and nonsmokers, by sex, region, age, and overall. Analyses are adjusted by age and stratified by study and sex. The horizontal lines (or widths of diamonds for overall results) show 95% confidence intervals (CIs). The probability values shown are for the test of interaction between systolic blood pressure and smoking status. The dashed (lower) and continuous (upper) lines represent present smokers and nonsmokers, respectively.

gestion of an interaction for CHD and ischemic stroke when restricting the present analysis to those studies with information on cigarette consumption, in agreement with an earlier study[11] which reported that the risk of ischemic stroke increased more strongly with higher dose of smoking among individuals with hypertension compared with those without. By contrast, another study[21] reported that the smoking dose-related risk for all strokes was similar for both those with and without hypertension.

Unlike CHD and ischemic stroke, the prevailing cause of hemorrhagic stroke is rupture resulting from fragility (including microaneurysms) of the intracerebral penetrating arteries caused by nonoptimal levels of BP or amyloid angiopathy.[31,32] This accounts for the stronger association between BP and hemorrhagic stroke risk compared with CHD, although the risk related to increased levels of BP is similar for ischemic and hemorrhagic stroke.[4,5] By contrast, the excess risk attributable to smoking for hemorrhagic stroke is less than it is for either CHD or ischemic stroke.[9–11] As regards a pathophysiological mechanism behind the interaction for hemorrhagic stroke observed in the present study, we can only speculate that smoking may promote the weakening of the intracranial blood vessels caused by high levels of BP or amyloid angiopathy. Only Thrift and colleagues[23] have examined the interaction between BP and smoking status for hemorrhagic stroke events. In this case-control study, a significant synergistic interaction was observed only in men, which is consistent with our findings. The sex-specific effect that we observed may have been a chance finding as a consequence of the few events among the smaller population of female smokers (n=14 031), compared with male smokers (n=196 930). The regional specificity may result from the difficulty in observing hemorrhagic stroke events due to a much smaller number participants and a lower event rate of hemorrhagic stroke in ANZ (204 events per 99 470 ANZ participants) compared with Asia (1411 events per 463 674

Asian participants). However, neither of these explanations would explain the age-specific significant effect, wherein the interaction only occurred among those aged 65 years or over: 883 events for <65 years and 762 events for ≥65 years.

The present study has some limitations. First, some cohorts in APCSC do not have information on other risk factors for CVD at baseline, restricting our ability to adjust for important covariates which may explain the observed interaction effects between BP and smoking. Serum total cholesterol, which is positively associated with CHD and ischemic stroke events, and inversely with hemorrhagic stroke events,[33] was available on 353 158 individuals; data on other potentially useful covariates was less common. However, adjustment for total cholesterol had negligible impact on any of the reported results (not shown). Second, we had limited data on daily dose of smoking and little information on how smoking status changed during follow-up, which did not allow any reliable analyses of follow-up smoking status comparable to our treatment of SBP. Third, the main analysis was not adjusted for antihypertensive medication status because of a lack of this information for more than 70% of participants, although the sensitivity analysis suggests that it may have little material impact on the results. Finally, there was lack of standardization of methods and procedures among the participating studies in APCSC, because the participating studies were originally independent of each other. For instance, only 56% of fatal and 65% of nonfatal strokes were objectively (using CT/MRI or autopsy findings) classified as ischemic or hemorrhagic in origin. The Hisayama study in Japan,[34] 1 of the APCSC participating studies, investigated the accuracy of diagnosis of each subtype of CVD using autopsies in the 1960s, 1970s, and 1980s. The accuracy of diagnosis was similar for ischemic and hemorrhagic stroke (confirmation rate 60% to 70%), which was better than the accuracy for CHD (46%).[34] Therefore, misclassification of stroke subtype may have introduced bias the extent of which would have varied across the studies.

In conclusion, we have shown that a combination of present smoking and nonoptimal levels of BP appears to have a synergistic impact on the risk of hemorrhagic stroke, at least among men and in the elderly, although the underlying pathophysiological mechanism is unclear, and we cannot exclude that similar synergism may occur among younger people and women. Furthermore, we cannot affirm the absence of interaction between BP and smoking for CHD and ischemic stroke. Further studies allowing for better verification of pathological types of stroke, better assessment of smoking status and other variables, and using a larger and more standardized dataset, are warranted to determine whether the interaction between BP and smoking really exists for each subtype of CVD, what mechanism explains the interaction, and how specific it is to demographic groups. Although quitting smoking and lowering BP are both crucial for prevention of CVD, combining the two could be expected to have extra beneficial effect on preventing hemorrhagic stroke. Thus, smoking cessation initiatives should be targeted more rigorously for hypertensive patients to prevent hemorrhagic stroke.

Appendix

The Asia Pacific Cohort Studies Collaboration

Executive Committee

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Sources of Funding

This project has received support from a National Health and Medical Research Council of Australia program grant (358395) and an unrestricted educational grant from Pfizer Inc. The sponsors had no influence on design, analysis, or interpretation of results, and took no part in the writing of this paper. C.M.M. Lawes is supported by a National Heart Foundation (New Zealand) Fellowship.

Disclosures

None.

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