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Adjustment for smoking does not alter the *FOXO3A* association with longevity

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Abstract Human longevity is a multifactorial phenotype influenced by both genetic and environmental factors. Despite its heritability of 25–32 %, the genetic background of longevity is as yet largely unexplained. Apart from *APOE* status, variation in the *FOXO3A* gene is the only confirmed genetic contributor to survival into old age. On the other hand, *FOXO3A* activity is known to be downregulated in various cancers, and the gene was recently identified as a novel deletion hotspot in human lung adenocarcinoma. In view of the strong association between smoking and lung cancer, we set out to explore whether smoking modifies the known association between *FOXO3A* variation and longevity. To this end, we conducted a case–control study in two different populations, drawing upon extensive collections of old-aged individuals and younger controls available to us (1,613 German centenarians/nonagenarians and 1,104 controls; 1,088 Danish nonagenarians and 736 controls). In the German sample, 21 single nucleotide polymorphisms (SNPs) from the *FOXO3A* gene region were genotyped, whereas 15 *FOXO3A* SNPs were analyzed in the Danish sample. Eight SNPs were typed in both populations. Logistic regression analysis revealed that adjustment for smoking does not systematically alter the association between *FOXO3A* variation and longevity in neither population. Our analysis therefore suggests that the said association is not largely due to the confounding effects of lung cancer.

Keywords Longevity · Aging · Smoking · Lung cancer · Confounder · Heritability

Carolin Däumer and Friederike Flachsbart contributed equally to this work.

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Introduction

Human longevity is a complex phenotype, and genetic factors have been estimated to account for ~30 % of the overall variation in adult lifespan (Christensen et al. 2006; Finch and Tanzi 1997; Gogele et al. 2011; Herskind et al. 1996; Hjelmborg et al. 2006; Ljungquist et al. 1998; Skytthe et al. 2003). As yet, however, variation in only two genes has been confirmed to influence

survival into old age, namely in those encoding apolipoprotein E (*APOE*), with *ε4* being a mortality factor in the elderly (Blanché et al. 2001; Christensen et al. 2006; Deelen et al. 2011; Nebel et al. 2011; Schachter et al. 1994), and transcription factor forkhead box O3A (*FOXO3A*). A comparatively modest association between *FOXO3A* polymorphisms and longevity has been observed in different populations (Anselmi et al. 2009; Flachsbart et al. 2009; Li et al. 2009; Pawlikowska et al. 2009; Soerensen et al. 2010; Willcox et al. 2008). However, the molecular mechanisms underlying this relationship still remain to be elucidated. Most of the longevity-associated *FOXO3A* single nucleotide polymorphisms (SNPs) analyzed so far are located in intronic regions, and the "functionally relevant" variants still have to be identified (Donlon et al. 2012; Flachsbart et al. 2012).

The FOXO3A protein is an evolutionarily conserved key regulator of the insulin-IGF1 signaling pathway (Hwangbo et al. 2004; Kenyon 2005; Kenyon et al. 1993; Lin et al. 1997; Ziv and Hu 2011). It also plays an important role in growth arrest, DNA repair, and apoptosis in response to DNA damage and oxidative stress (Furukawa-Hibi et al. 2002; Greer and Brunet 2005; Kops et al. 2002; Tran et al. 2002). Furthermore, FOXO3A has been implicated as a tumor suppressor (Fei et al. 2009; Hu et al. 2004; Mikse et al. 2010; Yang et al. 2008), and FOXO3A activity was consistently found to be downregulated in various cancers (Yang and Hung 2009; Greer and Brunet 2005). More recently, FOXO3A was also reported to stimulate a proapoptotic transcriptional program in response to a human lung carcinogen (Blake et al. 2010), and the *FOXO3A* gene region is a target of somatic deletion in lung adenocarcinoma (LAC) in both humans (Mikse et al. 2010) and mice (Herzog et al. 2009).

Since development of lung cancer clearly reduces an individual's life expectancy, the above association implies that the known influence of *FOXO3A* on longevity may be due, at least partially, to confounding by lung cancer. Such provisos are not uncommon in epidemiology, so that the allowance for possible confounders in the respective analyses has become mandatory to avoid false positive results. However, since lung cancer is not directly observable in a case–control study of longevity, we had to resort to smoking as a proxy for the disease (Doll et al. 2004; Dayan 1986; Liu et al. 1998; Warner et al. 1989), knowing that many smokers never develop lung cancer, and that smoking has many effects upon longevity that are unrelated to lung cancer (Fig. 1). This incongruence implies that the effects of smoking adjustment on the *FOXO3A*–longevity relationship can only partly reflect the relevance of lung cancer. This notwithstanding, if the association between *FOXO3A* and longevity was indeed confounded by lung cancer, then the same association would be expected to be notably stronger among smokers than among nonsmokers.

To address the above questions, we performed a case–control study in two populations, namely a German sample comprising 1,613 long-lived individuals (95–110 years) and 1,104 younger controls, and a Danish sample of 1,088 cases aged 92–93 years and 736 younger controls (Table 1). Individuals were classified as either smokers (ever smokers) or nonsmokers (never smokers). One caveat has to be taken into account in the present study, however, namely that cases and controls belong to different generations. Smoking behavior is known to have changed during the twentieth century (Benowitz et al. 2005; Franceschi and Bidoli 1999) so that "smoking" may have meant different things in the two groups. Nevertheless, as regards possible confounding by lung cancer, we think that use of such a "noisy" proxy was still valid. In view of the important role of *APOE* allele *ε4* in human longevity (Blanché et al. 2001; Christensen et al. 2006; Deelen et al. 2011; Nebel et al. 2011; Schachter et al. 1994), we also adjusted all analyses for the *APOE ε4* status.

Material and methods

Study population

We analyzed two case–control samples from two different populations (Table 1). The German sample included 1,613 unrelated old-aged individuals, with an age range of 95 to 110 years (median age 99 years). Some 27 % of these were male. Old-aged individuals were recruited from different geographic regions of Germany and included a subset of 748 centenarians (median age 101 years). The 1,104 controls were 60 to 75 years old (median age 67 years), and were matched for ancestry, sex, and geographic origin within the country. The recruitment of the German sample has been described in detail elsewhere (Nebel et al. 2005).

The Danish cohort comprised of 1,088 old-aged individuals (29 % male) born in 1905 (Nybo et al. 2001). The 736 younger controls were randomly selected from the study of middle-aged Danish twins (Skytthe et al.

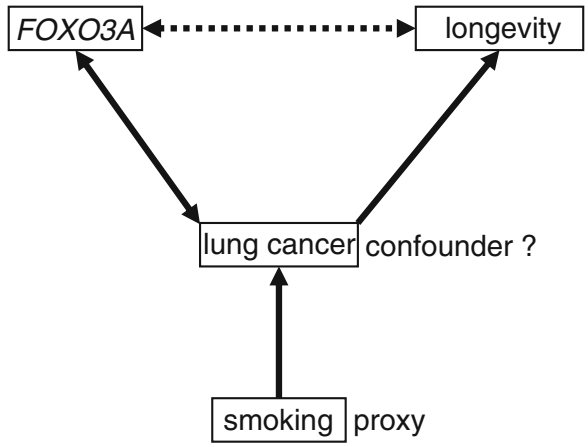

Fig. 1 Study setting. Associations between variables are depicted by *bold arrows*. A *dashed arrow* is used to indicate that the association between *FOXO3A* and longevity may be due partially to confounding by lung cancer. Smoking serves as a proxy for lung cancer. Note that cases and controls differ by both their life expectancy and generation membership

2002), but only one twin of each twin pair was included in the present study. In the controls, the sex ratio was approximately 1:1. At the time of interviewing, the old individuals were 92 to 93 years old (median age 93 years) and the younger controls were 46 to 54 years old (median age 50.5 years). The Danish study population also has been described in more detail elsewhere (Soerensen et al. 2010).

Genotyping

The German sample was genotyped on an automated platform (Hampe et al. 2001) using Taqman SNP genotyping assays (Life Technologies Corporation, Foster City, CA). A subset of the German sample and some SNPs have been analyzed before (Flachsbart et al. 2009), with six additional SNPs and 955

Table 1 Study samples

	Median age* (in years)	Total number	Smokers# (%)	Number of *FOXO3A* SNPs analyzed
German				
Old-aged individuals	99 (95–110)	1,613 Male: 436 (27.0 %) Female: 1,177 (73.0 %)	27.1	21
Controls	67 (60–75)	1104 Male: 283 (25.6 %) Female: 821 (74.4 %)	50.7	
Danish				
Old-aged individuals	93 (92–93)	1,088 Male: 313 (28.7 %) Female: 775 (71.2 %)	45.9	15
Controls	50.5 (46–54)	736 Male: 371 (50.4 %) Female: 365 (49.6 %)	62.6	
German and Danish				
Old-aged individuals	96 (92–110)	2,701 Male: 749 (27.7 %) Female:1,952 (72.3 %)	35.4	8
Controls	63 (46–75)	1,840 Male: 654 (35.5 %) Female:1,186 (64.5 %)	55.5	

* Age range is given in parenthesis; # ever smokers

additional individuals investigated here. Genotyping of the Danish sample was performed on the Illumina GoldenGate platform (Illumina Inc) (Steemers and Gunderson 2005; Soerensen et al. 2010). All samples and SNPs have been investigated before (Soerensen et al. 2010). In total, 21 *FOXO3A* SNPs were analyzed in the German sample (Table 2) and 15 SNPs were analyzed in the Danish sample (Table 3). Eight of the *FOXO3A* SNPs and the two *APOE* SNPs rs7412 and rs429358 were analyzed in both populations (Table 4).

Smoking information

For the German sample, information on smoking behavior was obtained via questionnaires. Individuals were asked whether they had ever smoked, whether they were recent smokers, for how many years they had smoked, and how many cigarettes they smoked per day. For the Danish sample, individuals were interviewed and classified as never, former, or current smokers. More detailed smoking information is provided in Supplementary Table S1.

Statistical analysis

Statistical analyses were performed using software R v.12.0 (Team RDC 2008). All tests were two-sided at the 5 % significance level. The genotyped SNPs were tested for Hardy–Weinberg equilibrium in the controls using an exact test as implemented in R-package *genetics* (Warnes et al. 2011). Associations between a SNP genotype and longevity were assessed for statistical significance by means of logistic regression analysis with and without interaction, followed by a Wald test (Wald 1943). SNP genotypes were coded by the dosage of the

Table 2 Association between longevity and *FOXO3A* SNP genotype. German sample (1,613 old-aged individuals, 1,104 controls)

No.	dbSNP ID	MAF controls (n=1104)	MAF cases (n=1613)	Risk allele	Unadjusted			Adjusted for smoking		
					P	OR	95 % CI	P	OR	95 % CI
1	rs2274776	0.490	0.486	–	0.899	1.009	0.881–1.154	0.928	1.007	0.871–1.164
2	rs1571631	0.465	0.462	–	0.847	1.013	0.885–1.160	0.986	1.001	0.866–1.158
3	**rs6911407**	**0.378**	**0.415**	**A**	**0.032**	**1.163**	**1.013–1.336**	**0.010**	**1.215**	**1.048–1.409**
4	**rs768023**	**0.382**	**0.415**	**G**	**0.049**	**1.149**	**1.001–1.319**	**0.016**	**1.201**	**1.035–1.392**
5	rs2802288	0.385	0.407	–	0.139	1.091	0.972–1.226	0.096	1.112	0.981–1.260
6	rs2883881	0.093	0.087	–	0.659	1.047	0.855–1.281	0.463	1.086	0.872–1.353
7	rs12200646	0.123	0.138	–	0.192	1.119	0.945–1.326	0.259	1.110	0.926–1.331
8	**rs2802290**	**0.387**	**0.417**	**G**	**0.083**	**1.130**	**0.984–1.298**	**0.025**	**1.185**	**1.022–1.375**
9	**rs2802292**	**0.382**	**0.413**	**G**	**0.062**	**1.141**	**0.993–1.310**	**0.043**	**1.165**	**1.005–1.351**
10	rs13220810	0.259	0.251	–	0.561	1.039	0.913–1.184	0.607	1.037	0.902–1.193
11	**rs2764264**	**0.304**	**0.338**	**C**	**0.039**	**1.164**	**1.007–1.346**	**0.019**	**1.203**	**1.030–1.405**
12	**rs7762395**	**0.153**	**0.175**	**A**	**0.092**	**1.174**	**0.974–1.416**	**0.023**	**1.266**	**1.034–1.551**
13	**rs13217795**	**0.297**	**0.328**	**C**	**0.048**	**1.160**	**1.001–1.345**	**0.020**	**1.206**	**1.030–1.412**
14	**rs9400239**	**0.295**	**0.331**	**T**	**0.028**	**1.180**	**1.018–1.369**	**0.007**	**1.245**	**1.062–1.460**
15	**rs3800231**	**0.287**	**0.327**	**A**	**0.010**	**1.215**	**1.047–1.410**	**0.002**	**1.290**	**1.100–1.513**
16	**rs4945816**	**0.289**	**0.316**	**C**	**0.062**	**1.126**	**0.994–1.276**	**0.031**	**1.160**	**1.014–1.326**
17	**rs4946936**	**0.291**	**0.321**	**G**	**0.045**	**1.133**	**1.003–1.279**	**0.024**	**1.162**	**1.020–1.324**
18	**rs1268170**	**0.347**	**0.377**	**G**	**0.060**	**1.147**	**0.994–1.323**	**0.017**	**1.204**	**1.033–1.403**
19	**rs473268**	**0.337**	**0.367**	**A**	**0.077**	**1.139**	**0.986–1.316**	**0.023**	**1.197**	**1.025–1.398**
20	**rs479744**	**0.199**	**0.229**	**T**	**0.020**	**1.225**	**1.032–1.454**	**0.005**	**1.304**	**1.085–1.567**
21	rs519007	0.186	0.183	–	0.798	1.023	0.860–1.217	0.865	1.016	0.844–1.223

MAF minor allele frequency, *Risk allele* "risk" allele for, attaining old age, *P p* value obtained from a risk allele-based case–control comparison using a Wald test, *OR* odds ratio for attaining old age, 95 % *CI* 95 % confidence interval. All analyses were adjusted for *APOE* status. In the combined sample, nationality was included as an additional influential variable. Nominally significant associations upon adjustment for smoking (p<0.05) are printed in bold

Table 3 Association between longevity and *FOXO3A* SNP genotype. Danish sample (1,088 old-aged individuals, 736 controls)

No.	dbSNP ID	MAF controls (*n*=736)	MAF cases (*n*=1,088)	Risk allele	Unadjusted			Adjusted for smoking		
					P	OR	95 % CI	P	OR	95 % CI
1	**rs9486902**	**0.178**	**0.203**	**A**	**0.057**	**1.192**	**0.881–1.154**	**0.048**	**1.205**	**1.002–1.450**
2	rs10499051	0.095	0.087	–	0.468	1.091	0.885–1.160	0.706	1.047	0.823–1.332
3	rs12206094	0.258	0.282	–	0.181	1.111	1.013–1.336	0.132	1.129	0.964–1.321
4	rs2802292	0.355	0.379	–	0.179	1.105	1.001–1.319	0.103	1.131	0.975–1.310
5	rs13220810	0.286	0.258	–	0.116	1.129	0.972–1.226	0.076	1.149	0.985–1.339
6	rs2764264	0.286	0.294	–	0.735	1.026	0.855–1.281	0.516	1.052	0.902–1.226
7	rs7762395	0.143	0.158	–	0.260	1.115	0.945–1.326	0.209	1.132	0.933–1.374
8	rs12207868	0.099	0.101	–	0.918	1.012	0.984–1.298	0.946	1.008	0.801–1.269
9	rs13217795	0.275	0.289	–	0.506	1.053	0.993–1.310	0.363	1.075	0.920–1.255
10	rs9400239	0.277	0.292	–	0.415	1.065	0.913–1.184	0.276	1.089	0.934–1.270
11	rs12212067	0.101	0.102	–	0.984	1.002	1.007–1.346	0.962	1.005	0.803–1.259
12	rs9398172	0.275	0.288	–	0.440	1.061	0.974–1.416	0.320	1.080	0.928–1.259
13	rs3800231	0.276	0.293	–	0.343	1.076	1.001–1.345	0.222	1.101	0.943–1.284
14	rs3800232	0.123	0.123	–	0.900	1.013	1.018–1.369	0.935	1.009	0.817–1.246
15	rs479744	0.191	0.211	–	0.149	1.133	1.047–1.410	0.068	1.175	0.989–1.397

MAF minor allele frequency, *Risk allele* "risk" allele for attaining old age, *P p* value obtained from a risk allele-based case–control comparison using a Wald test, *OR* odds ratio for attaining old age, 95 % *CI* 95 % confidence interval. All analyses were adjusted for *APOE* status. In the combined sample, nationality was included as an additional influential variable. Nominally significant associations upon adjustment for smoking ($p<0.05$) are printed in bold

respective "risk allele" for attaining old age, thereby assuming multiplicativity of the odds ratios and ensuring that reported odds ratios were always larger than unity. All SNP association analyses were adjusted for *APOE* genotype coded by the number of *APOE ε4* alleles ("dosage"). When German and Danish data were analyzed together, nationality was added as an additional influential variable.

Model selection involving all *FOXO3A* SNPs was impossible due to multicollinearity caused by

Table 4 Association between longevity and *FOXO3A* SNP genotype. Combined sample (2,701 old-aged individuals, 1,840 controls)

No.	dbSNP ID	MAF controls (*n*=1840)	MAF cases (*n*=2701)	Risk allele	Unadjusted			Adjusted for smoking		
					P	OR	95 % CI	P	OR	95 % CI
1	**rs2802292**	**0.368**	**0.396**	**G**	**0.023**	**1.123**	**1.016–1.241**	**0.009**	**1.150**	**1.036–1.276**
2	rs13220810	0.270	0.254	–	0.143	1.076	0.975–1.188	0.099	1.091	0.984–1.209
3	**rs2764264**	**0.295**	**0.316**	**C**	**0.084**	**1.096**	**0.988–1.217**	**0.031**	**1.127**	**1.011–1.256**
4	**rs7762395**	**0.148**	**0.166**	**A**	**0.046**	**1.145**	**1.002–1.308**	**0.012**	**1.197**	**1.041–1.377**
5	**rs13217795**	**0.286**	**0.308**	**C**	**0.060**	**1.107**	**0.996–1.231**	**0.021**	**1.139**	**1.020–1.272**
6	**rs9400239**	**0.286**	**0.311**	**T**	**0.033**	**1.122**	**1.010–1.248**	**0.007**	**1.164**	**1.042–1.300**
7	**rs3800231**	**0.281**	**0.310**	**A**	**0.013**	**1.144**	**1.029–1.272**	**0.002**	**1.190**	**1.065–1.329**
8	**rs479744**	**0.195**	**0.220**	**T**	**0.009**	**1.176**	**1.042–1.326**	**0.001**	**1.234**	**1.088–1.400**

MAF minor allele frequency, *Risk allele* "risk" allele for attaining old age, *P p* value obtained from a risk allele-based case–control comparison using a Wald test, *OR* odds ratio for attaining old age, 95 % *CI* 95 % confidence interval. All analyses were adjusted for *APOE* status. In the combined sample, nationality was included as an additional influential variable. Nominally significant associations upon adjustment for smoking ($p<0.05$) are printed in bold

linkage disequilibrium (LD). To avoid collinearity, we excluded SNPs in such a way that all remaining markers had pairwise r^2 values <0.80 (see Supplementary Figures S1–S3). LD plots were prepared with Haploview v4.2 (Barrett et al. 2005). The following *FOXO3A* SNPs were eventually taken into account in the following model selection: rs2274776, rs1571631, rs2883881, rs1220646, rs2802290, rs2802292, rs13220810, rs2764264, rs7762395, rs13217795, rs473268, rs479744, and rs519007 for the German sample; rs9486902, rs10499051, rs12206094, rs2802292, rs13220810, rs7762395, rs12207868, rs3800232, and rs479744 for the Danish sample; and rs2802292, rs13220810, rs2764264, rs7762395, rs13217795, and rs479744 for the combined sample. Starting from the full model, we performed backward selection that kept only influential variables with a p value <0.05 in the final model.

Results

Association of *APOE* and smoking with longevity

As was to be expected, smoking (ever versus never) and *APOE ε4* dosage both had a strong impact upon longevity (Table 5), with estimated odds ratios of 0.36 and 0.42, respectively, in the German sample and of 0.51 and 0.58 in the Danish sample. No statistically significant interaction was observed between the two factors.

Table 5 Association between longevity and either *APOE* status or smoking

	Simple logistic regression analysis		Multiple logistic regression analysis	
	P	OR	P	OR
German				
APOE	$<2\times10^{-16}$	0.427	$<2\times10^{-16}$	0.424
Smoking status	$<2\times10^{-16}$	0.361	$<2\times10^{-16}$	0.379
Danish				
APOE	3.2×10^{-8}	0.580	2.9×10^{-8}	0.495
Smoking status	2.9×10^{-12}	0.505	2.0×10^{-8}	0.572

P p values were obtained using a Wald test, *OR* odds ratio for attaining old age; simple logistic regression analysis, both influential variables were analyzed separately; multiple logistic regression analysis, both influential variables were analyzed jointly. No statistically significant interaction was observed in any of the analyses; *APOE* number of *APOE ε4* alleles; smoking status, ever versus never smokers with never smokers as reference category

Association between *FOXO3A* and longevity, with and without adjustment for smoking

We analyzed the association between longevity and genetic variation in the *FOXO3A* gene region for 21 SNPs in the German sample and 15 SNPs in the Danish sample. Eight of the SNPs that had been genotyped in both groups were analyzed in both samples combined. All SNPs were in Hardy–Weinberg equilibrium in the control samples, and all analyses were adjusted for *APOE ε4* dosage. In the German sample, eight SNPs (rs6911407, rs768023, rs2764264, rs13217795, rs9400239, rs3800231, rs4946936, and rs479744) were significantly associated with longevity (Table 2). In the Danish sample, no significant association between a SNP and longevity was detected (Table 3). In the combined sample, five SNPs (rs2802292, rs7762395, rs9400239, rs3800231, and rs479744) were found to be significantly associated with longevity (Table 4). After adjustment for smoking, all significant genotype–phenotype relationships remained significant (Fig. 2). In addition, some of the SNPs acking an association in the unadjusted analysis became nominally significant after adjustment for smoking as follows: rs2802290, rs2802292, rs7762395, rs4945816, rs1268170, and rs473268 in the German sample, rs2764264 and rs13217795 in the combined sample set, and rs9486902 in the Danish sample (Tables 2, 3, and 4).

Model selection with and without adjustment for smoking

In order to assess the joint effects upon longevity of *FOXO3A* variation, *APOE* genotype, nationality, and smoking, allowing for a possible correlation between these variables (e.g., due to linkage disequilibrium), we performed two types of logistic regression analysis with backward model selection. Initial model M1 included only the *FOXO3A* SNPs and *APOE ε4* dosage (plus nationality, when appropriate), whereas model M2 also included smoking. In the German sample, the same *FOXO3A* SNPs occurred in final models M1 and M2 (Table 6), namely rs1200646 and rs479744. Moreover, *APOE* and smoking (in the case of model M2) were also included in the models. Final model M1 for the Danish sample did not include any *FOXO3A* SNP, but only *APOE ε4* dosage. Final model M2 included *APOE*, *FOXO3A* SNP rs13220810, and smoking. For the combined sample, both final models included *FOXO3A* SNP rs479744

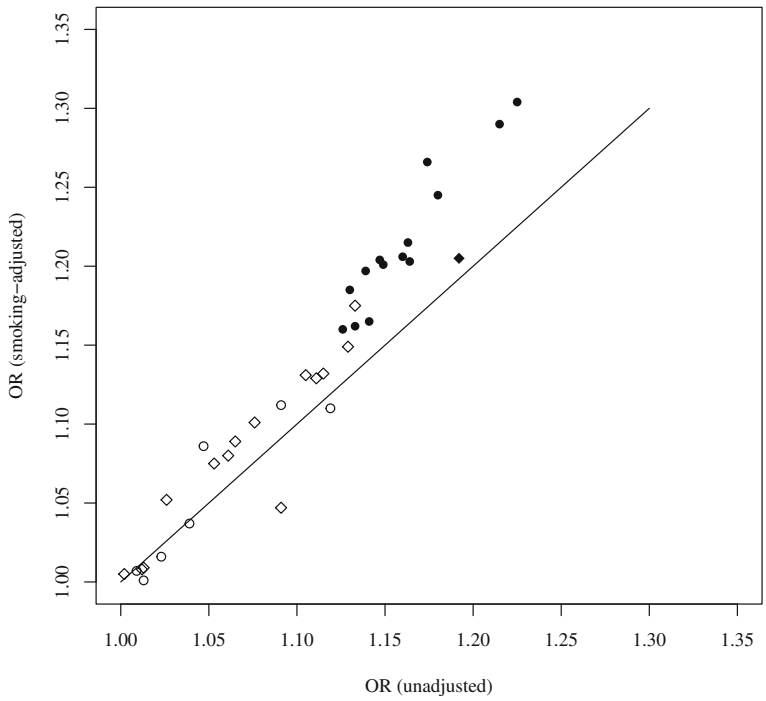

Fig. 2 *FOXO3A* odds ratios for attaining old age, with and without adjustment for smoking. Odds ratios were obtained by logistic regression analyses of longevity and *FOXO3A* SNP genotype, with (*vertical axis*) and without smoking adjustment (*horizontal axis*). *Circles* German sample; *diamonds* Danish sample. Significant associations are marked by *filled symbols*. All analyses were adjusted for *APOE* genotype

and *APOE ε4* dosage. Apart from smoking, nationality was also found to be a significantly influential variable in model M2 (but not in M1). To test the robustness of the model selection, we added smoking to final model M1. All previously selected influential variables remained statistically significant in all three samples (German, Danish, and combined). No statistically significant pairwise interaction between the different influential variables, particularly not between smoking and any *FOXO3A* genotype, was observed.

Robustness of results

The above results were based upon the use of two smoking categories, namely "ever" and "never". We also adopted alternative classifications of smoking status,

Table 6 Logistic regression model selection

Sample	Final model without smoking (M1)			M1+Smoking			Final model with smoking (M2)		
	Influential variables	P	OR	Influential variables	P	OR	Influential variables	P	OR
German	rs1200646	0.021	1.289	rs1200646	0.031	1.288	rs1200646	0.031	1.288
	rs479744	0.019	1.242	rs479744	0.006	1.314	rs479744	0.006	1.314
	APOE	8.5×10^{-12}	2.278	*APOE*	7.1×10^{-11}	2.351	*APOE*	7.1×10^{-11}	2.351
				Smoking	1.3×10^{-14}	2.402	Smoking	1.3×10^{-14}	2.402
Danish	*APOE*	1.2×10^{-8}	1.801	*APOE*	1.1×10^{-8}	1.827	rs13220810	0.036	1.041
				Smoking	1.1×10^{-12}	2.110	*APOE*	1.3×10^{-8}	1.821
							Smoking	2.7×10^{-13}	2.136
Combined	rs479744	0.014	1.170	rs47944	0.003	1.219	rs479744	0.002	1.225
	APOE	$<2\times10^{-16}$	1.937	*APOE*	$<2\times10^{-16}$	1.932	*APOE*	$<2\times10^{-16}$	1.982
				Smoking	$<2\times10^{-16}$	2.115	Smoking	$<2\times10^{-16}$	2.220
							Nationality	1.8×10^{-4}	1.331

P p values were obtained using a Wald test, *OR* odds ratio for attaining old age, nationality was encoded as 1 for German and 0 for Danish, *APOE* number of *APOE ε4* alleles, *smoking status* ever versus never smokers with never smokers as reference category

particularly for the German sample, where more information on smoking behavior was available. For example, we considered "never and former" versus "current" and "smoking for more than 5 years" versus "never". The results of these analyses turned out to be very similar to those obtained with the original classification (data not shown). We also performed a sex-stratified analysis, with results both for males and females that were very similar to the results of the non-stratified analysis (see Supplementary Tables S2 and S3). In the Danish males, however, one SNP (rs13220810) ceased to show a significant association with longevity upon adjustment for smoking.

Discussion

Variation in the *FOXO3A* gene is not only associated with longevity (Anselmi et al. 2009; Flachsbart et al. 2009; Li et al. 2009; Pawlikowska et al. 2009; Soerensen et al. 2010; Willcox et al. 2008) but also plays a role in the etiology of various neoplasias, including lung cancer (Donlon et al. 2012; Greer and Brunet 2005; Herzog et al. 2009; Mikse et al. 2010; Willcox et al. 2008; Yang and Hung 2009). In the present study, we therefore set out to investigate whether the association observed between *FOXO3A* and longevity may have been due, at least partially, to the confounding effects of lung cancer. Unfortunately, lung cancer mortality is difficult, if not impossible, to address in retrospective case–control studies. However, since smoking is strongly associated with lung cancer (Dayan 1986; Doll et al. 2004; Liu et al. 1998; Warner et al. 1989), we thought that a combined analysis of longevity and *FOXO3A* variation using smoking as a proxy for lung cancer still appeared well warranted. If confounding by the disease played an important role, such an analysis should have yielded a smaller or even absent residual effect of *FOXO3A* variation on aging, or a significant interaction between *FOXO3A* genotype and smoking. Contrary to this expectation, our study revealed an unaltered genotype–phenotype association after adjustment for smoking in both single and multiple SNP analyses. The latter ultimately invoked only smoking and one or two *FOXO3A* SNPs as significantly influential variables, and no interaction with smoking became apparent.

Interestingly, both the number and the magnitude of significant *FOXO3A* SNP associations differed considerably between the German and the Danish samples analyzed here, with the German sample showing the stronger associations. This discrepancy is potentially explicable by the fact that the association between *FOXO3A* and longevity is much stronger in centenarians than in nonagenarians (Flachsbart et al. 2009). In the Danish sample, the age range was only 92 to 93 years, compared to 95 to 110 years in the Germans, who even included a subset of 748 centenarians. Moreover, in the first report of an association between *FOXO3A* and longevity in the Danish population, the association was observed only for males and only using specific modes of inheritance (Soerensen et al. 2010). Such differences notwithstanding, smoking adjustment did not alter the said genotype–phenotype relationship in neither population samples.

Our study was not intended to investigate the effect of smoking on longevity, but rather addressed the possible confounding effects of lung cancer. Smoking prevalence varied considerably in the past and therefore differed between our cases and controls as well simply because the two groups belonged to different generations (Deutsches Krebsforschungszentrum 2008; Peto et al. 2000). Notably, the quality of smoking has also changed, for example, by a trend towards so-called "light" cigarettes that stimulate smokers to inhale more deeply (Benowitz et al. 2005; Franceschi and Bidoli 1999). This means that our proxy for lung cancer may have been somewhat imprecise. However, several aspects seem to support the general validity of our conclusion that the *FOXO3A*-longevity association is not strongly confounded by lung cancer. First, we obtained similar results in two different populations, and with several SNPs. Second, although smoking prevalence and smoking habits are known to differ considerably between males and females, we obtained similar results in a sex-stratified as in the non-stratified analyses. Finally, a lack of an effect of smoking adjustment was consistently seen with all smoking classifications employed here.

In our study, the number of smokers was considerably higher in the Danish sample than in the German sample (Table 1). This was true for the controls, but even more so for the old-aged individuals. Not surprisingly, a stronger association between smoking and longevity was, therefore, seen in the German sample. There are several possible explanations for this population difference in smoking prevalence. Danish individuals were recruited in a cohort study and were observed over a long period of time. Therefore, their

smoking behavior could be recorded more precisely than in the German sample, which followed a case–control design with the usual drawback of recall bias, especially at exceptionally old age. However, the difference in smoking prevalence did not qualitatively change the effect of smoking adjustment on the association between *FOXO3A* variation and longevity.

Our study followed a case–control design, which is known to be inferior to a cohort design in many respects. Most importantly, case–control studies are liable to recall bias so that environmental exposure data may lack the accuracy of analogous information from direct follow-up. Moreover, as was mentioned in the introduction, the relationship between an exposure and a phenotype may become confounded by age if the typology of the exposure, in this case smoking, has changed over time. However, since the association between *FOXO3A* variation and longevity only becomes apparent for extremely old age (≥95 years of age), prospective studies are difficult because of the extremely low prevalence of the phenotype (0.01–0.02 %, Perls 2006) and the long follow-up period required. Therefore, case–control studies almost inevitably have become the most popular design for the investigation of longevity (Deelen et al. 2013). Moreover, as has been pointed out above, our study was not intended to investigate the effect of smoking on longevity per se, which would be difficult to do in a case–control design anyway. Instead, we were interested in the possible confounding effect of lung cancer (with smoking used as a proxy), which should be detectable in case–control studies as well. Finally, it must be emphasized that the association between *FOXO3A* variation and longevity was originally detected (Willcox et al. 2008) and subsequently confirmed in case–control studies (Anselmi et al. 2009; Flachsbart et al. 2009; Li et al. 2009; Pawlikowska et al. 2009; Soerensen et al. 2010). Only one study was prospective in nature (Soerensen et al. 2010). Bearing in mind the methodological limitations of case–control studies and their possible impact on statistical power, the question whether an association was confounded by a given candidate can, thus, only sensibly be clarified using the same study design.

In the future, it would be interesting to explore the potential relationship between genetic variation in *FOXO3A* and various cancers from the viewpoint that the former is a potential tumor suppressor (Fei et al. 2009; Hu et al. 2004; Mikse et al. 2010; Yang et al. 2008). So far, little is known about the link between germline *FOXO3A* variation and a predisposition to cancer (Campa et al. 2011). For example, deletions in the *FOXO3A* gene region in LAC were explored in somatic cancer cells only (Mikse et al. 2010). Moreover, an investigation of the role of *FOXO3A* variation in cancers other than LAC might provide further independent insight into the importance of *FOXO3A* for human aging and longevity (Donlon et al. 2012).

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