Supplementary Information

The Cytochrome P450 Slow Metabolizers CYP2C9*2 and CYP2C9*3 Directly Regulate Tumorigenesis via Reduced Epoxyeicosatrienoic Acid Production

Lindsay N. Sausville, Mahesha Gangadhariah, Manuel Chiusa, Shaojun Mei, Shouzuo Wei, Roy Zent, James M. Luther, Megan M. Shuey, Jorge H Capdevila, John R Falck, F Peter Guengerich, Scott M Williams, Ambra Pozzi

gene	forward	reverse	PCR conditions
CYP2C9*2 (R144C)	5'-GAGGAGCATTGAGGACTGT GTTCAAGAGGAAGC-3'	5'-GCTTCCTCTTGAACACAGT CCTCAATGCTCCTC-3'	95 °C x 60 seconds, 1 cycle; 95 °C x 60 seconds; 60 °C x 60 seconds; 68 °C x 10 min, 18 cycles; 72 °C x 10 seconds, 1 cycle.
CYP2C9*3 (I359L)	5'-CGAGGTCCAGAGATACCTT GACCTTCTCCCCAC-3'	5'-GTGGGGAGAAGGTCAAGGT ATCTCTGGACCTCG-3'	95 °C x 60 seconds, 1 cycle; 95 °C x 60 seconds; 60 °C x 60 seconds; 68 °C x 10 min, 18 cycles; 72 °C x 10 seconds, 1 cycle.

Supplementary Table S1

Generation of CYP2C9* variants cDNAs. Sequence of primers and PCR conditions used to generate CYP2C9*2 (R144C) and CYP2C9*3 (I359L) cDNAs.

SNP	**Chr**	**Location**	**Genic Location**	**Major/Minor Allele**	**MAF**
rs7085745	10	96682514	Upstream	T/C	0.207
rs4918758	10	96697252	Upstream	T/C	0.353
rs2253635	10	96700537	Intron	A/G	0.411
rs12772884	10	96700630	Intron	T/A	0.440
rs1799853 (2C9*2)	10	96702047	Missense	C/T	0.116
rs9332172	10	96731788	Intron	A/G	0.178
rs1057910 (2C9*3)	10	96741053	Missense	A/C	0.063
rs1934967	10	96741426	Intron	C/T	0.230
rs11188130	10	96767767	Downstream	A/C	0.268
rs1934975	10	96769769	Downstream	T/C	0.322
rs11188133	10	96772015	Downstream	A/G	0.415

Supplementary Table S2

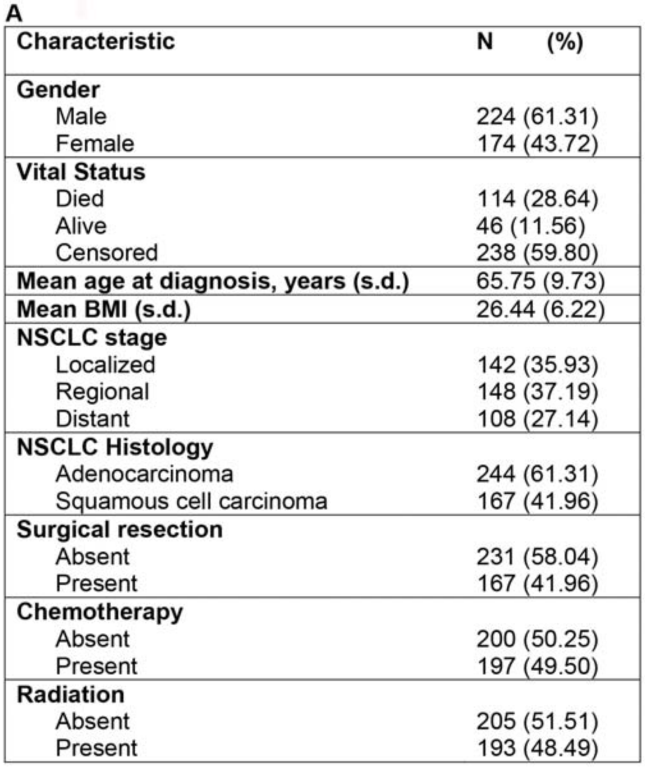
SNPs genotyped in the BioVU NSCLC study. For each SNP in the table the location is indicated by the chromosome and base pair position according to GRCh37.p13.

Chr, Chromosome; MAF, Minor allele frequency

SNP	**% Missing**	**Genotype Counts**			**HWE *p*-value**
		0	**1**	**2**	
rs7085745	0	248	135	15	0.647
rs4918758	0	162	191	45	0.380
rs2253635	0	132	205	61	0.216
rs12772884	0	116	214	68	0.084
*rs1799853 (2C9*2)*	0	312	80	6	0.631
rs9332172	0.252	269	115	13	0.864
*rs1057910 (2C9*3)*	0.252	347	50	0	0.387
rs1934967	0	230	153	15	0.118
rs11188130	0	208	167	23	0.200
rs1934975	0	187	16	45	0.421
rs11188133	0	135	196	67	0.837

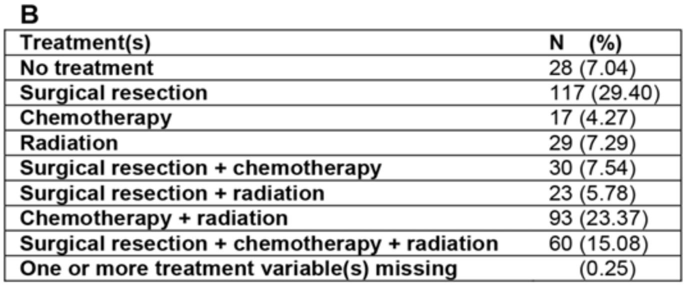
Supplementary Table S3

Quality control measures of SNPs in the BioVU NSCLC study. From the population (n =398) the following was determined for each SNP genotyped: percent missing, genotype counts, and Hardy-Weinberg (HWE) p-values.



A

Characteristic	N (%)
Gender	
Male	224 (61.31)
Female	174 (43.72)
Vital Status	
Died	114 (28.64)
Alive	46 (11.56)
Censored	238 (59.80)
Mean age at diagnosis, years (s.d.)	65.75 (9.73)
Mean BMI (s.d.)	26.44 (6.22)
NSCLC stage	
Localized	142 (35.93)
Regional	148 (37.19)
Distant	108 (27.14)
NSCLC Histology	
Adenocarcinoma	244 (61.31)
Squamous cell carcinoma	167 (41.96)
Surgical resection	
Absent	231 (58.04)
Present	167 (41.96)
Chemotherapy	
Absent	200 (50.25)
Present	197 (49.50)
Radiation	
Absent	205 (51.51)
Present	193 (48.49)



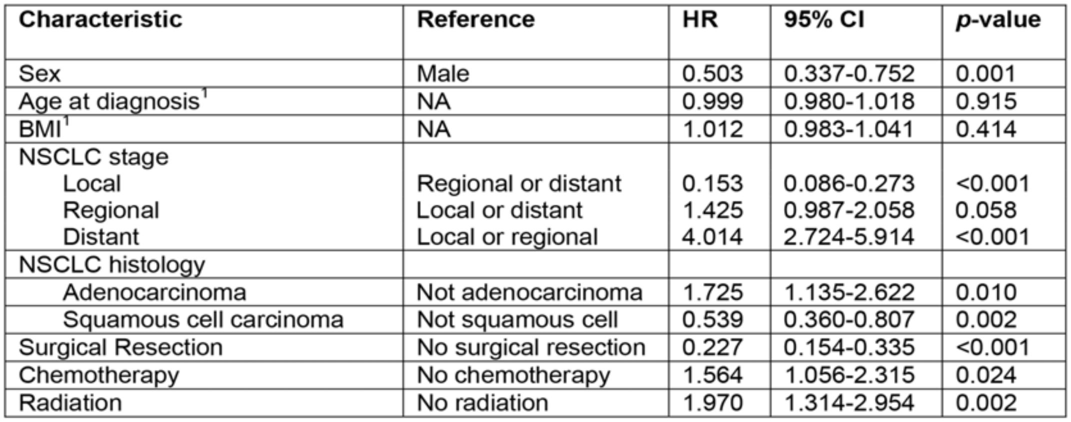
B

Treatment(s)	N (%)
No treatment	28 (7.04)
Surgical resection	117 (29.40)
Chemotherapy	17 (4.27)
Radiation	29 (7.29)
Surgical resection + chemotherapy	30 (7.54)
Surgical resection + radiation	23 (5.78)
Chemotherapy + radiation	93 (23.37)
Surgical resection + chemotherapy + radiation	60 (15.08)
One or more treatment variable(s) missing	(0.25)

Supplementary Table S4

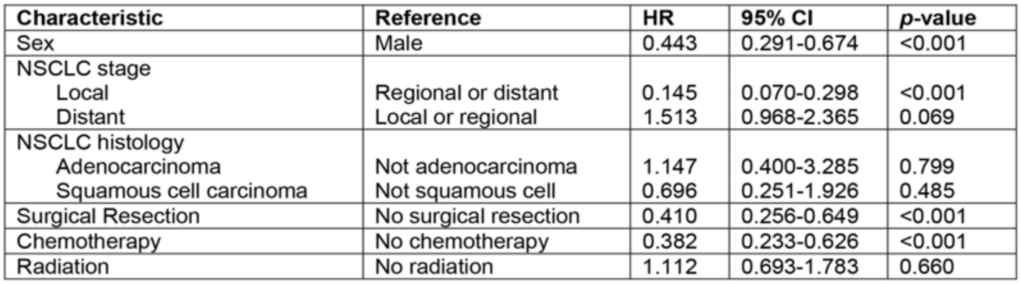
Clinical and demographic description of NSCLC cases. **A,** Clinical and demographic variable distribution in the NSCLC cases included for survival analyses. Only NSCLC cases with stage data available were included. As adenocarcinoma and squamous cell carcinoma contain the majority of NSCLC cases, no other histology was reported in this study. Additionally, overlap was permitted for histology. One individual was missing chemotherapy treatment information, but otherwise information was complete for all individuals unless otherwise noted. **B**, Distribution of combinations of treatments.

A



Characteristic	Reference	HR	95% CI	*p*-value
Sex	Male	0.503	0.337-0.752	0.001
Age at diagnosis[1]	NA	0.999	0.980-1.018	0.915
BMI[1]	NA	1.012	0.983-1.041	0.414
NSCLC stage				
Local	Regional or distant	0.153	0.086-0.273	<0.001
Regional	Local or distant	1.425	0.987-2.058	0.058
Distant	Local or regional	4.014	2.724-5.914	<0.001
NSCLC histology				
Adenocarcinoma	Not adenocarcinoma	1.725	1.135-2.622	0.010
Squamous cell carcinoma	Not squamous cell	0.539	0.360-0.807	0.002
Surgical Resection	No surgical resection	0.227	0.154-0.335	<0.001
Chemotherapy	No chemotherapy	1.564	1.056-2.315	0.024
Radiation	No radiation	1.970	1.314-2.954	0.002

B



Characteristic	Reference	HR	95% CI	*p*-value
Sex	Male	0.443	0.291-0.674	<0.001
NSCLC stage				
Local	Regional or distant	0.145	0.070-0.298	<0.001
Distant	Local or regional	1.513	0.968-2.365	0.069
NSCLC histology				
Adenocarcinoma	Not adenocarcinoma	1.147	0.400-3.285	0.799
Squamous cell carcinoma	Not squamous cell	0.696	0.251-1.926	0.485
Surgical Resection	No surgical resection	0.410	0.256-0.649	<0.001
Chemotherapy	No chemotherapy	0.382	0.233-0.626	<0.001
Radiation	No radiation	1.112	0.693-1.783	0.660

Supplementary Table S5

A, The association of clinical and demographic variables with survival was examined with the Kaplan-Meier estimator. Hazard ratios (HR) and 95% confidence intervals (95% CI) were determined with a Cox proportional model to show the direction of effect. **B**, The Cox proportional hazard test was used to determine to hazard ratio, 95% confidence interval, and p-value in an adjusted model including all variables that associated with survival as determined by the Kaplan-Meier estimator. This adjusted model was used to assess if a variable independently associated with survival, or if the association was driven by its association with another variable predicting survival.

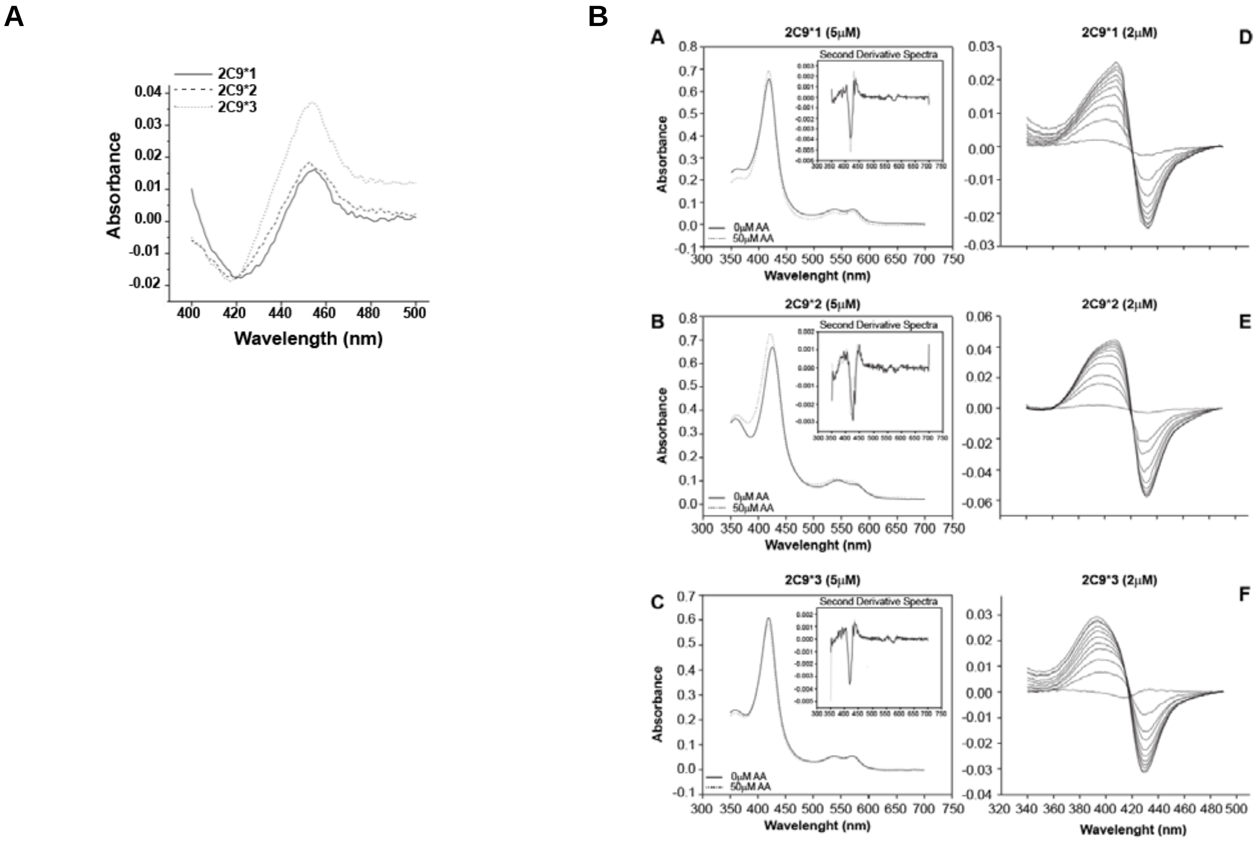
[1] indicates a continuous variable where the association with survival, including the p-value, was tested using the Cox proportional model.

NSCLC, non-small cell lung cancer; BMI, body mass index.

SNP	**KM *p*-value**
rs7085745	0.646
rs4918758	0.396
rs2253635	0.889
rs12772884	0.906
*rs1799853 (2C9*2)*	0.118
rs9332172	0.141
*rs1057910 (2C9*3)*	0.086
rs1934967	0.835
rs11188130	0.391
rs1934975	0.835
rs11188133	0.826
*Collapsed 2C9*2 or *3*	0.015

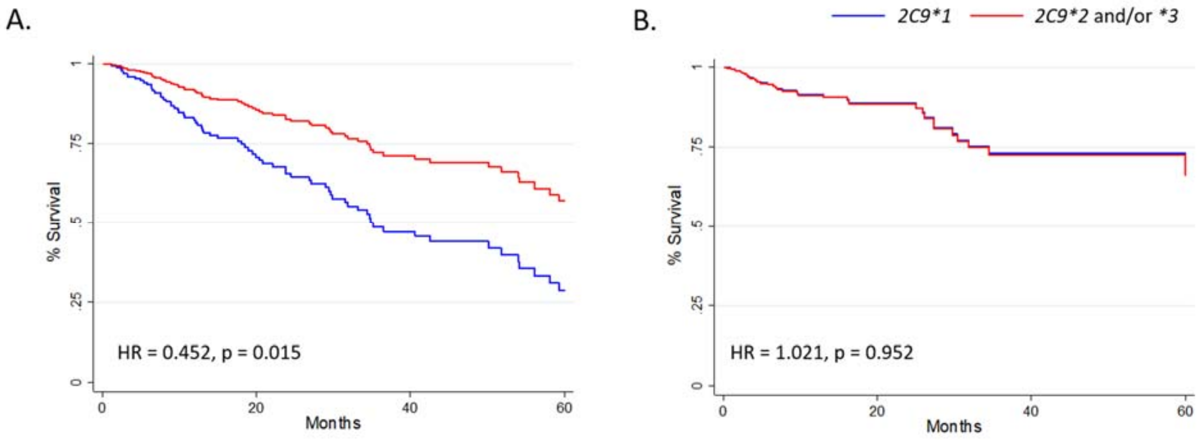
Supplementary Table S6

Description of genotypes in the NSCLC cases. Initial association of *CYP2C9* SNPs assessed by the Kaplan-Meier estimator.



Supplementary Figure S1

A, Ferrous-carbon monoxide vs. ferrous difference spectra of CYP2C9* variants. Reduction to ferrous iron was achieved by the addition of $Na_2S_2O_4$ as described by Omura and Sato (17). The concentrations of CYP2C9*1, CYP2C9*2, and CYP2C9*3 were 0.16, 0.16, and 0.24 μM, respectively. **B**, Spectral analysis of effects of arachidonic acid on CYP2C9* variants. **A-C**, Absolute spectra of CYP2C9* variants with or without a saturating concentration of AA (50 μM). The second derivative spectra ($d^2A/d\lambda^2$) are shown in the insets. **D-F**, Titrations of CYP2C9* variants (2.0 μM) with AA (0-100 μM). Two cuvettes containing CYP2C9* proteins were balanced to obtain a baseline, which was recorded. Increasing concentrations of AA (in ethanol) were added to the sample cuvette, and at each point an equivalent volume of the solvent was added to the reference cuvette. The spectrum was recorded following each set of additions, and the data absorbance spectral maxima (390 nm) and minima (420 nm) were plotted vs. AA concentration and fit to hyperbolic plots, used to calculate K_d values. The K_d values calculated in parts D-F were 5.7 ± 0.3 μM, 5.3 ± 0.4 μM, and 9.1 ± 0.5 μM, for CYP2C9*1, CYP2C9*2, and CYP2C9*3, respectively.



Supplementary Figure S2

*CYP2C9*2/*3* associates with improved survival in NSCLC receiving platinum chemotherapy. Five-year survival curves are shown for subjects receiving (**A)** and not receiving (**B**) platinum chemotherapy. Stratified analysis, on the basis of receiving platinum-based chemotherapy, were conducted with Cox proportional hazard models to assess the association of hypomorphic *CYP2C9* alleles with NSCLC survival adjusted for NSCLC stage, resection, non-platinum chemotherapy, and sex in NSCLC subjects. Within subjects receiving platinum chemotherapy, 54 individuals were available with either *CYP2C9*2* or *CYP2C9*3,* while a total of 73 individuals with either allele were available in subjects not receiving platinum chemotherapy.