

might regulate the clearance of deleterious mitochondria with new chemicals which target this pathway to maintain cell integrity and subsequently suppress cell death. However, further studies are required to elucidate the exact pathomechanism and develop effective therapies for hereditary PD and sporadic PD.

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Association of *LRRK2* exonic variants with susceptibility to Parkinson's disease: a case-control study

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Summary

Background The leucine-rich repeat kinase 2 gene (*LRRK2*) harbours highly penetrant mutations that are linked to familial parkinsonism. However, the extent of its polymorphic variability in relation to risk of Parkinson's disease (PD) has not been assessed systematically. We therefore assessed the frequency of *LRRK2* exonic variants in individuals with and without PD, to investigate the role of the variants in PD susceptibility.

Methods *LRRK2* was genotyped in patients with PD and controls from three series (white, Asian, and Arab-Berber) from sites participating in the Genetic Epidemiology of Parkinson's Disease Consortium. Genotyping was done for exonic variants of *LRRK2* that were identified through searches of literature and the personal communications of consortium members. Associations with PD were assessed by use of logistic regression models. For variants that had a minor allele frequency of 0.5% or greater, single variant associations were assessed, whereas for rarer variants information was collapsed across variants.

Findings 121 exonic *LRRK2* variants were assessed in 15 540 individuals: 6995 white patients with PD and 5595 controls, 1376 Asian patients and 962 controls, and 240 Arab-Berber patients and 372 controls. After exclusion of carriers of known pathogenic mutations, new independent risk associations were identified for polymorphic variants in white individuals (M1646T, odds ratio 1.43, 95% CI 1.15–1.78; $p=0.0012$) and Asian individuals (A419V, 2.27, 1.35–3.83; $p=0.0011$). A protective haplotype (N551K-R1398H-K1423K) was noted at a frequency greater than 5% in the white and Asian series, with a similar finding in the Arab-Berber series (combined odds ratio 0.82, 0.72–0.94; $p=0.0043$). Of the two previously reported Asian risk variants, G2385R was associated with disease (1.73, 1.20–2.49; $p=0.0026$), but no association was noted for R1628P (0.62, 0.36–1.07; $p=0.087$). In the Arab-Berber series, Y2189C showed potential evidence of risk association with PD (4.48, 1.33–15.09; $p=0.012$).

Interpretation The results for *LRRK2* show that several rare and common genetic variants in the same gene can have independent effects on disease risk. *LRRK2*, and the pathway in which it functions, is important in the cause and pathogenesis of PD in a greater proportion of patients with this disease than previously believed. These results will help discriminate those patients who will benefit most from therapies targeted at *LRRK2* pathogenic activity.

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Introduction

Parkinson's disease (PD) is generally thought of as a late-onset sporadic disorder. Nevertheless, genetic insights are helping to define the molecular causes of PD and have provided new models for the development of neuroprotective interventions. Mutations in the leucine-rich repeat kinase 2 gene (*LRRK2*) are now recognised as the most common genetic determinant of familial and sporadic PD.¹ *LRRK2* has 51 exons and encodes the 2527 aminoacid protein LRRK2, which has five conserved domains, including a Roc (Ras in complex proteins, Rab GTPase) domain and a catalytic core common to both tyrosine and serine-threonine kinases.

Pathogenic *LRRK2* variability has been identified by sequencing probands with familial parkinsonism, with results confirmed and occasionally extended within community or clinically-based patient-control series.^{2–6} Seven definite pathogenic *LRRK2* mutations (encoding LRRK2 N1437H, R1441C, R1441G, R1441H, Y1699C, G2019S, and I2020T) have been described.^{7,8} These mutations can be relatively common in patients from some ethnic origins, but are rare in ethnically matched controls. LRRK2 R1441G has been identified in more than 8% of patients with PD originating from the Basque region of northern Spain,⁹ and LRRK2 G2019S has been reported in 30% of Arab-Berber patients

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with PD.^{10,11} *LRRK2* polymorphisms with more than 1% minor allele frequency have also been associated with PD in Asia, with the estimated attributable risk often dependent on ethnic origin. *LRRK2* R1628P and G2385R have each been recorded in 3–4% of individuals who are of Chinese descent and roughly double the risk of PD.^{12–15}

However, most *LRRK2* variants have not been systematically studied. *LRRK2* might harbour more variants that are important determinants of PD pathogenicity and clinical risk. To address this possibility, with the Genetic Epidemiology of Parkinson's Disease (GEO-PD) Consortium, we assessed the frequency of *LRRK2* exonic variants in people with and without PD, and assessed the role of the variants in disease susceptibility.

Methods

Participants and procedures

All 35 GEO-PD sites (hospitals and centres), representing 22 countries and six continents, were invited to participate in this study. Patients were diagnosed by use of either the Gelb or the UK Parkinson's Disease Society Brain Bank criteria (the exclusion criterion of more than one affected relative was not included).^{16,17} Controls at each site were healthy individuals who were not related to the patients; not all controls were given a detailed neurological examination but all were asked about any previous diagnosis or family history of a neurological disorder. All biological samples were gathered after ethics approval had been obtained from the Mayo Clinic Institutional Review Board Committee, and were used in accordance with the terms of the written informed consent provided by the participants.

LRRK2 exonic variants were identified through searches of available literature up to April 1, 2010, from personal communications with consortium members, and from in-house sequencing studies that had identified novel variants (unpublished data; table 1). DNA was sourced from blood and was stored in a –20°C freezer. All samples were de-identified with an anonymous code from each site and only a minimal clinical dataset. Data were collected in batches but analysed as a single dataset. Genotyping was done on a MassArray iPLEX platform (Sequenom, San Diego, CA, USA) at the Mayo Clinic neurogenetics laboratory, FL, USA (except for the groups from Paris, France, and Antwerp, Belgium, who supplied genotype data and positive control genomic DNA^{2,3}); all primer sequences are provided in the webappendix pp 1–4). Eight iPLEX variant combinations were used to incorporate 123 *LRRK2* coding variants (table 1). Positive control DNA was run for each variant; in the absence of a positive genomic control DNA, a synthetic positive control DNA sequence was generated by use of mismatch-primer PCR. A χ^2 test followed by Bonferroni correction was used to test for deviation from the Hardy–Weinberg equilibrium (HWE) in controls for

	Exon	Accession number	cDNA	Aminoacid	Domain
chr12:38905228	1	..	28G>A	E10K	..
chr12:38905349	1	rs2256408	149G>A	R50H	..
chr12:38905627	2	rs72546335	155C>T	S52F	..
chr12:38905696	2	rs75054132	224G>A	A75A	..
chr12:38915703	4	rs33995463	356T>C	L119P	..
chr12:38915711	4	rs41286468	364T>C	L122L	..
chr12:38918058	5	rs10878245	457T>C	L153L	..
chr12:38918147	5	rs35517158	546A>G	K182K	..
chr12:38920612	6	rs112794616	632C>T	A211V	..
chr12:38920663	6	rs56108242	683G>C	C228S	..
chr12:38923625	7	rs28365216	713A>T	N238I	..
chr12:38923737	7	rs72546315	824C>T	H275H	..
chr12:38929923	8	rs17490713	867T>C	N289N	..
chr12:38929949	8	rs57355477	893T>C	A298A	..
chr12:38929992	8	rs41286466	936G>T	A312A	..
chr12:38931342	9	rs78501232	1000G>A	E334K	..
chr12:38931397	9	rs36016791	1055delC	A352fsX357	..
chr12:38931430	9	rs72546336	1088A>G	N363S	..
chr12:38931438	9	rs113065049	1096G>A	V366M	..
chr12:38933053	11	rs34594498	1256C>T	A419V	..
chr12:38937411	12	rs35847451	1383C>T	S461S	..
chr12:38939594	13	rs75711334	1464A>T	L488L	..
chr12:38939673	13	rs34090008	1543insG	P514fsX529	..
chr12:38943875	14	rs35328937	1561A>G	R521G	..
chr12:38943944	14	rs79996249	1630A>G	K544E	..
chr12:38943967	14	rs7308720	1653C>G	N551K	..
chr12:38954669	15	rs77424631	1647G>A	G558G	..
chr12:38958002	17	rs78154388	1987T>C	S663P	..
chr12:38958037	17	rs72546319	2022A>C	V674V	..
chr12:38958213	17	rs35611877	2198insA	L708fsX718	Ankyrin
chr12:38958223	18	..	2134A>G	M712V	Ankyrin
chr12:38958236	18	..	2147C>T	A716V	Ankyrin
chr12:38958256	18	rs10878307	2167A>G	I723V	Ankyrin
chr12:38963966	19	rs34410987	2264C>T	P755L	Ankyrin
chr12:38964080	19	rs35173587	2378G>T	R793M	Ankyrin
chr12:38964130	19	rs72546337	2428A>G	I810V	Ankyrin
chr12:38964183	19	rs76890302	2481T>C	S827S	Ankyrin
chr12:38967530	20	..	2611A>G	K871E	..
chr12:38973693	21	rs58559150	2769G>C	Q923H	..
chr12:38973713	21	..	2789A>G	Q930R	..
chr12:38974935	22	rs17519916	2830G>T	D944Y	..
chr12:38974962	22	rs7966550	2857T>C	L953L	..
chr12:38975535	23	rs75148313	2918G>A	S973N	..
chr12:38975635	23	rs113217062	3018A>G	I1006M	LRR
chr12:38975638	23	rs55783828	3021C>T	S1007S	LRR
chr12:38978415	24	rs111341148	3200G>A	R1067Q	LRR
chr12:38978502	24	rs76535406	3287C>G	S1096C	LRR
chr12:38978548	24	rs78365431	3333G>T	Q1111H	LRR
chr12:38978557	24	rs35808389	3342A>G	L1114L	LRR
chr12:38979194	25	rs34805604	3364A>G	I1222V	LRR
chr12:38979281	25	rs74985840	3451G>A	A1151T	LRR
chr12:38979324	25	..	3494T>C	L1165P	LRR

(Continues on next page)

	Exon	Accession number	cDNA	Aminoacid	Domain
(Continued from previous page)					
chr12:38982935	26	..	3574A>G	I1192V	LRR
chr12:38984073	27	rs72546324	3647A>G	H1216R	LRR
chr12:38984109	27	rs80179604	3683G>C	S1228T	LRR
chr12:38984109	27	rs60185966	3683G>T	S1228I	LRR
chr12:38985860	28	rs4640000	3784C>G	P1262A	LRR
chr12:38988536	29	rs77018758	3960G>C/T	R1320S	..
chr12:38988550	29	rs72546338	3974G>A	R1325Q	..
chr12:38988687	29	rs17466213	4111A>G	I1371V	Roc
chr12:38988701	29	rs28365226	4125C>A	D1375E	Roc
chr12:38989178	30	rs7133914	4193G>A	R1398H	Roc
chr12:38989214	30	rs72546327	4229C>T	T1410M	Roc
chr12:38989243	30	rs113589830	4258G>A	D1420N	Roc
chr12:38989254	30	rs11175964	4269G>A	K1423K	Roc
chr12:38989275	30	rs111435410	4290C>T	A1430A	Roc
chr12:38989294	30	rs74163686	4309A>C	N1437H	Roc
chr12:38990503	31	rs33939927	4321C>T	R1441C	Roc
chr12:38990503	31	rs33939927	4321C>G	R1441G	Roc
chr12:38990504	31	rs34995376	4322G>A	R1441H	Roc
chr12:38990505	31	rs112998035	4323C>T	R1441R	Roc
chr12:38990506	31	..	4324G>C	A1442P	Roc
chr12:38990519	31	rs74681492	4337C>T	P1446L	Roc
chr12:38990530	31	rs111501952	4348G>A	V1450I	Roc
chr12:38990569	31	rs35363614	4387insA	R1462fsX1468	Roc
chr12:38990584	31	..	4402A>G	K1468E	Roc
chr12:38990630	31	rs113431708	4448G>A	R1483Q	Roc
chr12:38994045	32	rs35507033	4541G>A	R1514Q	COR
chr12:38994128	32	rs33958906	4624C>T	P1542S	COR
chr12:38994170	32	rs17491187	4666C>A	L1556I	COR
chr12:38995335	33	rs721710	4793T>A	V1598E	COR
chr12:39000067	34	..	4838T>C	V1613A	COR
chr12:39000101	34	rs1427263	4872C>A	G1624G	COR
chr12:39000112	34	rs33949390	4883G>C	R1628P	COR
chr12:39000140	34	rs11176013	4911A>G	K1637K	COR
chr12:39000166	34	rs35303786	4937T>C	M1646T	COR
chr12:39000168	34	rs11564148	4939T>A	S1647T	COR
chr12:39000188	34	rs111503579	4959A>G	L1653L	COR
chr12:39001183	35	rs35801418	5096A>G	Y1699C	COR
chr12:39001350	35	rs79909111	5163A>G	S1721S	COR
chr12:39002106	36	rs11564176	5173C>T	R1725X	COR
chr12:39002116	36	..	5183G>T	R1728L	COR
chr12:39002116	36	rs145364431	5183G>A	R1728H	COR
chr12:39002455	37	rs111910483	5385G>T	L1795F	COR
chr12:39002527	37	rs10878371	5457T>C	G1819G	COR
chr12:39003324	38	..	5605A>G	M1869V	COR
chr12:39003325	38	rs35602796	5606T>C	M1869T	COR
chr12:39003329	38	..	5610G>T	L1870F	COR
chr12:39003339	38	..	5620G>T	E1874X	COR
chr12:39015100	39	rs77428810	5822G>A	R1941H	MAPKKK
chr12:39020430	41	..	6016T>C	Y2006H	MAPKKK
chr12:39020449	41	rs34015634	6035T>C	I2012T	MAPKKK
chr12:39020469	41	rs34637584	6055G>A	G2019S	MAPKKK

(Continues on next page)

each site. Direct DNA sequencing was used to confirm genotyping for all variants with a frequency of less than 0.3% ($n < 50$ carriers).

Statistical analysis

All analyses were undertaken separately for the patients in the white, Asian, and Arab-Berber series. For common variants with a minor allele frequency of 0.5% or greater, single variant associations with PD were assessed by use of fixed-effects logistic regression models, in which genotypes were dichotomised as presence versus absence of the minor allele (dominant model), because *LRRK2* mutations cause an autosomal dominantly inherited form of PD and homozygotes for many of the variants are rare; additive models were also assessed. Models were adjusted for site in the white and Asian series. Sensitivity of results to the use of random-effects models was also assessed.¹⁸ Odds ratios (ORs) and 95% CIs were estimated. Between-site heterogeneity was assessed with likelihood ratio tests for variant by site interaction in a logistic regression analysis, and also by estimation of the I^2 statistic (a measure of the proportion of total variation in ORs between sites due to heterogeneity beyond chance).¹⁹

For variants with a minor allele frequency of less than 0.5% (rare variants), although we estimated the proportion of carriers separately in patients and controls, no statistical tests were used to evaluate associations with PD because of insufficient power. Instead, we collapsed information for rare variants, acknowledging that this has the potential limitation of mixing groups of variants with protective and risk effects, and evaluated the association between the presence of any rare variant and PD in a logistic regression analysis adjusted by site.²⁰ In an exploratory analysis, when collapsing data across variants, we also used the Sorts Intolerant From Tolerant (SIFT) prediction program²¹ to assess only those substitutions predicted to be not tolerated.

Haplotype analysis was done by use of score tests for association with adjustment for site;²² haplotypes of less than 0.5% frequency were not assessed. Any patient with a copy of the minor allele for any of the pathogenic variants that were noted in the study population (R1441C, R1441H, or G2019S) was excluded from all disease-association analyses to prevent confounding by the pathogenic variants; these patients were not excluded for any other portion of the analysis. Linkage disequilibrium between variants was assessed by use of r^2 values in study controls, separately for each series. Single variant associations with age at onset were assessed with linear regression models, adjusting for site in the white and Asian series; regression coefficients and 95% CIs were estimated.

We adjusted for multiple testing by use of the single-step minP method,²³ with 10 000 within-site permutations of outcome labels to assess the level of significance that controls the family-wise error rate at

5%. After this adjustment, in the logistic regression disease-association analysis $p \leq 0.0033$ was judged to be significant in the white series and $p \leq 0.0038$ in the Asian series, whereas in the linear regression age at onset association analysis $p \leq 0.0035$ was judged to be significant in the white series and $p \leq 0.0037$ in the Asian series. The adjusted significance cutoff levels differed between the white and Asian series because of the different number of tests undertaken in each series, and the different correlation structures between variants within them. For the fairly small Arab-Berber series, no adjustment for multiple testing was made, and as such the results were judged to be exploratory. All statistical analyses were done by use of SAS software (version 9.2) or S-Plus (8.0.1).

Role of the funding source

The funding agencies did not play any part in the design of the study, collection, analysis, or interpretation of data, writing of the report, or the decision to submit the report for publication. The principal investigators (OAR and MJF) had access to all the data in this study. The corresponding author had final responsibility for the decision to submit.

Results

Data were gathered from June, 2008, to October, 2010. 23 sites from the GEO-PD Consortium, representing 15 countries and five continents, agreed to participate in this study and contributed clinical data from 8611 patients with PD and 6929 controls. We studied individuals in three series: white (6995 patients and 5595 controls), Asian (1376 patients and 962 controls), and Arab-Berber (240 patients and 372 controls). Table 2 shows the demographics for each series, and webappendix p 5 shows the sample size breakdown for each site. 123 *LRRK2* variants were selected for genotype analysis, but two (R793M and L2466H) did not assay by use of iPLEX and were dropped from the study. The other 121 variants were genotyped in the entire patient-control series ($n=15\,540$); genotyping was successful in all individuals. Call rates for all genotypes in the series were greater than 95%. Deviation from HWE in the controls for each site (all $p > 0.05$) was noted for *LRRK2* N2081D in the Norwegian series and was attributable to two patients with a rare homozygous genotype; all patients were retained in the analysis. However, N289N and P1262A were excluded from the analysis of the Arab-Berber series because of significant variation from HWE due to an increased number of rare minor allele homozygotes, which might have been attributable to the consanguineous nature of the population.

Four of 121 *LRRK2* exonic variants were nonsense, 89 missense, and 28 silent. 48 variants, including four of the seven known pathogenic mutations, were not identified in the 15 540 patients and controls. For most of

	Exon	Accession number	cDNA	Aminoacid	Domain
(Continued from previous page)					
chr12:39020473	41	rs35870237	6059T>C	I2020T	MAPKKK
chr12:39020505	41	rs78029637	6091A>T	T2031S	MAPKKK
chr12:39026899	42	rs111739194	6187delCTCTA	L2063X	MAPKKK
chr12:39026953	42	rs33995883	6241A>G	N2081D	MAPKKK
chr12:39028521	43	rs10878405	6324G>A	E2108E	MAPKKK
chr12:39028553	43	rs12423862	6356C>T	P2119L	MAPKKK
chr12:39031648	44	rs111691891	6422C>T	T2141M	..
chr12:39031736	44	rs34869625	6510C>A	G2170G	WD40
chr12:39031792	44	rs35658131	6566A>G	Y2189C	WD40
chr12:39036195	46	rs12581902	6782A>T	N2261I	WD40
chr12:39043509	48	rs113511708	7067C>T	T2356I	WD40
chr12:39043595	48	rs34778348	7153G>A	G2385R	WD40
chr12:39043597	48	rs33962975	7155A>G	G2385G	WD40
chr12:39043610	48	rs79546190	7168G>A	V2390M	WD40
chr12:39044912	49	rs78964014	7183G>A	E2395K	WD40
chr12:39044916	49	rs111272009	7187insGT	T2356fsX2360	WD40
chr12:39044919	49	rs3761863	7190C>T	M2397T	WD40
chr12:39044953	49	rs60545352	7224G>A	M2408I	WD40
chr12:39047081	50	..	7397T>A	L2466H	WD40
chr12:39047119	50	rs55633591	7435A>G	N2479D	WD40

Chr12=chromosome 12. Roc=Ras in complex. COR=C-terminal of Ras. MAPKKK=mitogen-activated protein kinase kinase kinase. LRR=leucine-rich repeat.

Table 1: LRRK2 exonic variants investigated in the study

	Patients	Controls
White series	n=6995	n=5595
Age (years)	69 (12; 18–107)	65 (15; 19–107)
Men	4036 (58%)	2669 (48%)
Age at onset (years)	58 (12; 18–96)	NA
Asian series	n=1376	n=962
Age (years)	63 (13; 20–91)	59 (11; 23–98)
Men	681 (49%)	319 (33%)
Age at onset (years)	54 (12; 20–89)	NA
Arab-Berber series	n=240	n=372
Age (years)	66 (12; 27–87)	58 (11; 31–92)
Men	116 (48%)	190 (51%)
Age at onset (years)	57 (13; 20–82)	NA

Data are mean (SD; range) or number (%), unless otherwise indicated. Information about sex was not available for six patients and eight controls in the Asian series, and 16 patients and 249 controls in the white series. Information about age was not available for eight patients and eight controls in the Asian series, 482 patients and 289 controls in the white series, and six patients and four controls in the Arab-Berber series. Information about the age at onset was not available for 14 patients in the Asian series and 801 patients in the white series. 71 controls in the Taiwan case-control series overlapped with a previous study of R1628P.¹⁵ NA=not applicable.

Table 2: Characteristics of participants

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the variants, the pair-wise linkage disequilibrium was weak ($r^2 < 0.3$), with higher values noted with D' because of the low minor allele frequency for many of these variants (webappendix pp 6–17).

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	Aminoacid	White series				Asian series				Arab-Berber series			
		MA	MAF	OR (95% CI)	p value	MA	MAF	OR (95% CI)	p value	MA	MAF	OR (95% CI)	p value
rs2256408	R50H	G	+	+	+	G	1.7%	2.05 (0.82-5.14)	0.13
rs10878245	L153L	T	39.6%	0.98 (0.91-1.06)	0.57	C	31.2%	1.04 (0.88-1.23)	0.65	C	47.1%	0.81 (0.55-1.19)	0.28
rs34594498	A419V	T	+	+	+	T	1.9%	2.27 (1.35-3.83)	0.0011
rs7308720	N551K	G	6.7%	0.88 (0.79-0.98)	0.025	G	11.9%	0.73 (0.60-0.89)	0.0017	G	8.0%	0.83 (0.49-1.39)	0.47
rs10878307	I723V	G	7.4%	0.94 (0.84-1.04)	0.23	G	1.1%	1.36 (0.74-2.49)	0.32	G	9.0%	1.09 (0.68-1.75)	0.71
rs34410987	P755L	T	0.6%	0.56 (0.27-1.18)	0.13
rs58559150	Q923H	C	+	+	+	C	0.9%	0.62 (0.13-2.99)	0.55
rs7966550	L953L	C	12.8%	0.98 (0.90-1.07)	0.66	C	17.6%	0.80 (0.66-0.95)	0.012	C	12.4%	0.92 (0.60-1.41)	0.70
rs77018758	R1320S	T	1.2%	1.20 (0.69-2.11)	0.51
rs17466213	I1371V	G	+	+	+	G	+	+	+	G	0.5%	4.45 (0.81-24.56)	0.086
rs7133914	R1398H	A	6.6%	0.89 (0.80-0.99)	0.034	A	11.5%	0.73 (0.59-0.89)	0.0020	A	8.7%	1.00 (0.61-1.64)	1.00
rs11175964	K1423K	A	6.6%	0.83 (0.74-0.92)	0.0006	A	11.5%	0.75 (0.62-0.92)	0.0064	A	5.4%	0.42 (0.21-0.86)	0.011
rs35507033	R1514Q	A	0.9%	1.13 (0.85-1.49)	0.41	A	+	+	+
rs33958906	P1542S	T	2.8%	0.90 (0.77-1.06)	0.21	T	1.0%	2.27 (0.72-7.13)	0.16
rs1427263	G1624G	C	34.7%	1.06 (0.98-1.14)	0.15	A	46.7%	0.92 (0.77-1.11)	0.40	C	31.7%	0.96 (0.67-1.39)	0.84
rs33949390	R1628P	C	+	+	+	C	1.2%	0.62 (0.36-1.07)	0.087
rs11176013	K1637K	A	45.0%	1.02 (0.94-1.11)	0.60	G	44.6%	0.96 (0.80-1.16)	0.68	A	46.0%	1.07 (0.70-1.63)	0.76
rs35303786	M1646T	C	1.6%	1.43 (1.15-1.78)	0.0012	C	+	+	+
rs11564148	S1647T	A	29.9%	0.93 (0.86-1.00)	0.048	A	28.3%	0.97 (0.82-1.15)	0.73	A	27.6%	0.81 (0.55-1.19)	0.29
rs10878731	G1819G	T	45.2%	1.06 (0.98-1.15)	0.16	C	43.3%	0.99 (0.83-1.19)	0.95	T	46.2%	1.07 (0.70-1.64)	0.75
rs33995883	N2081D	G	2.6%	1.24 (1.05-1.47)	0.013	G	+	+	+	G	4.7%	0.92 (0.49-1.73)	0.79
rs10878405	E2108E	A	31.4%	0.96 (0.89-1.03)	0.27	A	29.6%	1.01 (0.85-1.20)	0.92	A	28.1%	0.75 (0.51-1.10)	0.14
rs35658131	Y2189C	G	+	+	+	G	1.1%	4.48 (1.33-15.09)	0.012
rs3477838348	G2385R	A	3.3%	1.73 (1.20-2.49)	0.0026
rs33962975	G2385G	G	15.7%	0.97 (0.89-1.06)	0.49	G	1.8%	0.96 (0.62-1.49)	0.85	G	8.4%	1.14 (0.7-1.83)	0.60
rs3761863	M2397T	C	34.4%	1.06 (0.98-1.14)	0.17	C	43.9%	0.88 (0.73-1.05)	0.16	C	39.8%	1.33 (0.85-2.07)	0.21

ORs and p values result from logistic regression models, where adjustment was made for the site in the Asian and white series. ORs correspond to the presence of the MA. After adjustment for multiple testing, p<0.0038 was judged to be significant in the Asian series, and p<0.0033 was judged to be significant in the white series. No adjustment for multiple testing was made in the Arab-Berber series, for which p<0.05 was judged to be significant. MA=minor allele. MAF=MA frequency. OR=odds ratio. +=a variant with a MAF of less than 0.5% and therefore not included in the logistic regression analysis. ..=a variant not noted in the series.

Table 3: Common single LRRK2 variant associations with Parkinson's disease

Table 3 shows the results of the disease-association analysis of single *LRRK2* variants. In the white series, significant associations with PD were noted for K1423K and M1646T. Figure 1 shows the country-specific ORs and 95% CIs for the risk factor M1646T. The between-site heterogeneity was low for M1646T ($I^2=0\%$, $p=0.44$) and moderate for K1423K ($I^2=34\%$, $p=0.069$) in the white series.

In the Asian series, significant associations with PD were noted for *LRRK2* A419V, N551K, R1398H, and G2385R (table 3). Figure 2 and figure 3 show the country-specific ORs and 95% CIs for A419V and G2385R, and for the N551K-R1398H-K1423K haplotype; between-site heterogeneity was very low for each of these associations in the Asian series (all $I^2=0\%$, all $p\geq 0.42$, webappendix p 18). Notably, *LRRK2* R1628P was not associated with PD in the Asian series (table 3), with a non-significant protective effect noted for this variant in the Taiwanese series (minor allele frequency 3.8%, OR 0.56, 95% CI 0.32–1.01; $p=0.054$). Although not significant, the predicted risk effect for R1628P was noted in the South Korean series, particularly at the Seoul site (0.2%, 2.47, 0.28–22.15; $p=0.42$). R1628P was not noted in the Japanese series. The previously suggested association of S1647T with PD in Asian populations¹⁴ was not supported by the results of our study (0.97, 0.82–1.15; $p=0.73$).

In an exploratory analysis of the small Arab–Berber series, significant associations ($p\leq 0.05$, without correction for multiple testing) with PD were noted for K1423K and Y2189C (table 3). Larger Arab–Berber series are needed to confirm these associations.

For patients with available information (95%), results for the analysis of the association of single variants with disease in each series remained similar after adjustment for age and sex (webappendix p 19) and by use of an additive model (webappendix p 20). Effect sizes were also similar after simultaneous adjustment for other variants that were significantly associated with PD in a particular series, and after adjustment for R1628P in the Asian series in which a previous association had been shown (webappendix p 21), providing evidence that these associations are independent of one another. With a random-effects model for the white and Asian series, results were generally similar though slightly weaker (webappendix p 18) than those obtained with a fixed-effects model.

Haplotype analysis showed a significant overall association with disease in the series of white ($p=0.0016$) and Asian ($p=2\times 10^{-24}$) individuals, but was non-significant in the Arab–Berber series ($p=0.056$). Haplotype associations seemed to be attributable to the variants independently implicated in disease (webappendix pp 22–24). When the three series were assessed together, *LRRK2* N551K, R1398H, and K1423K, which are in strong linkage disequilibrium and constitute a common (>5% frequency) haplotype, were associated with a

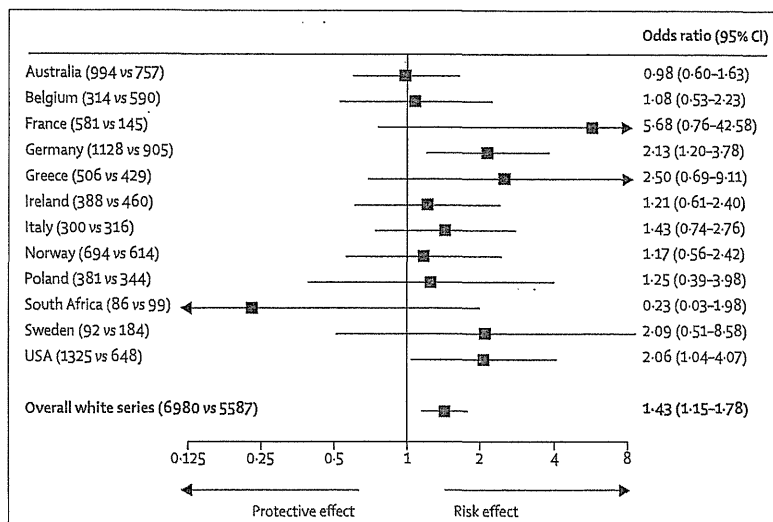


Figure 1: Forest plot of *LRRK2* variant M1646T in individuals with versus without Parkinson's disease in the white series

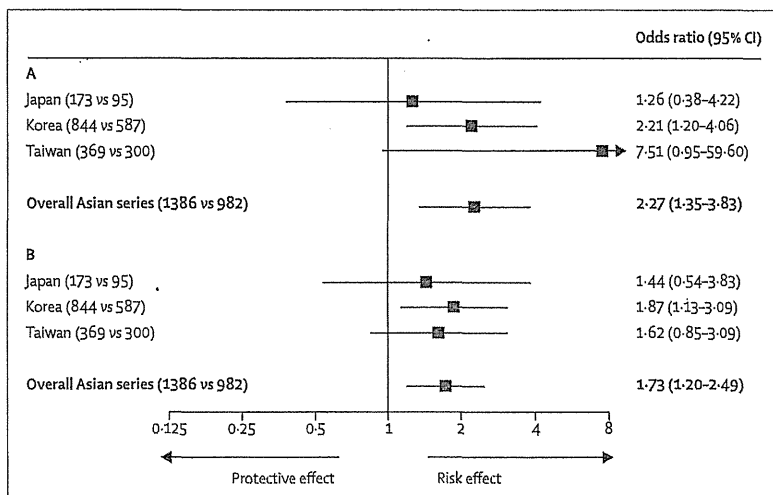


Figure 2: Forest plots of *LRRK2* variants A419V (A) and G2385R (B) in individuals with versus without Parkinson's disease in the Asian series

protective effect (combined OR 0.82, 95% CI 0.72–0.94; $p=0.0043$; figure 3).

Results of all common single variant associations with age at onset are shown on webappendix p 25. We did not identify any associations that withstood multiple testing correction in the white and Asian series. In the Arab–Berber series, L153L was associated with age at onset roughly 4 years earlier ($p=0.038$), which needs confirmation in larger samples.

Table 4 provides a descriptive summary of rare variants (minor allele frequency <0.5%) in patients and controls in each series. The pathogenic variant R1441H was noted in an Asian patient, R1441C in only ten patients from the white series, and G2019S in all three series (table 4). The

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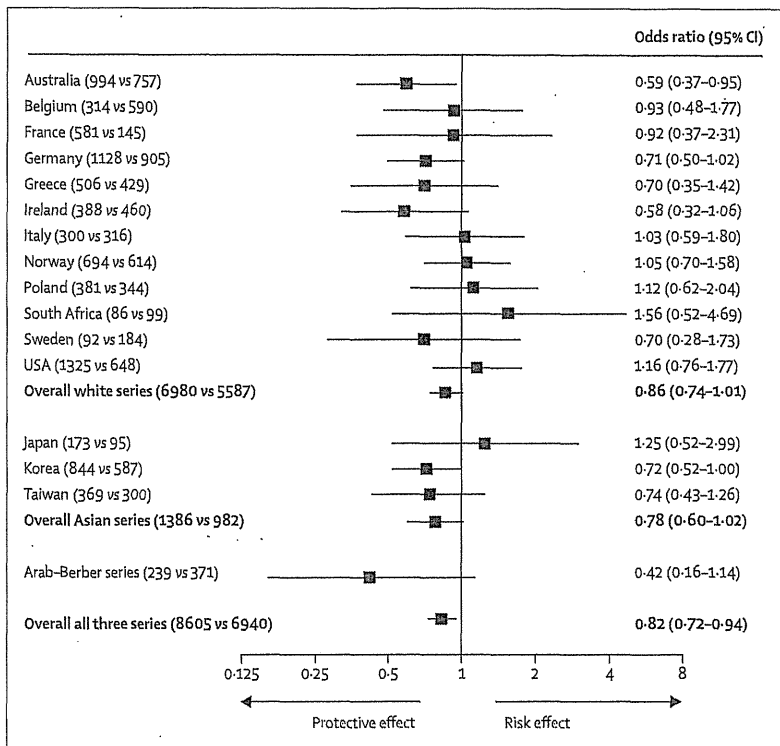


Figure 3: Forest plot of protective LRRK2 haplotype N551K-R1398H-K1423K in individuals with versus without Parkinson's disease in the white, Asian, and Arab-Berber series

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median age of the eight control carriers of G2019S was 64 years (range 48–76 years). Due to the strong confounding potential of these three variants on disease-association analyses, any patient with a copy of these risk alleles was excluded from the analysis. Other possible rare risk variants (E334K, R1325Q, and T1410M) and protective variants (A221V and A1151T) with differences in frequency between patients with PD and controls were noted. When data for all rare variants were combined, the presence of any rare variant was not associated with PD in the white series (OR 1.01, 95% CI 0.81–1.25; $p=0.95$), Asian series (1.03, 0.57–1.85; $p=0.92$), or Arab-Berber series (0.78, 0.28–2.20; $p=0.64$). Additionally, no association was noted in the white series (0.89, 0.55–1.43; $p=0.62$), Asian series (1.05, 0.37–2.99; $p=0.93$), or Arab-Berber series (no PD cases, two <1% controls, Fisher's exact $p=1.00$) when the data were combined only for those variants predicted by use of the SIFT program to be not tolerated.²⁴ Webappendix p 26 provides a summary of variants for which there were no carriers in any of the three series.

Discussion

The results of our study, one of the largest so far of the genetics of PD, show that a single gene, LRRK2, harbours many rare and common variants that confer susceptibility to PD in diverse populations (panel). Although population stratification is an inherent caveat

of this type of large-scale collaborative effort (and a potential limitation of the present study in the absence of genome-wide population control markers), these findings exemplify the confluence and independent effects of rare and common variations on gene loci that have a major effect in shaping both familial and sporadic disease.

About a third of variants we assessed were not identified in any study participant. These included four previously documented pathogenic mutations (LRRK2 N1437H, R1441G, Y1699C, and I2020T), showing that they are rare mutations in the population samples we assessed. 26 variants were recorded at a frequency greater than 0.5% in any of the three series, and only 13 were noted at a frequency greater than 0.5% in all three series. This finding draws attention to the importance of studying genetic variability in large samples and in different ethnic groups, because frequencies and genetic effects might vary substantially.²⁶

The newly identified associations warrant further discussion. M1646T in the COR (C-terminal of Ras) domain of LRRK2 was identified in the white series, and the effect was consistent in many countries (figure 1). This variant was not identified in participants of Asian descent and was rare in the series of Arab-Berber participants. LRRK2 A419V was consistently more common in patients than in controls in Asian sites (figure 2). Although we cannot exclude the possibility of a non-coding element in linkage disequilibrium, the N-terminal region of the protein seems functionally relevant to disease development. LRRK2 M1646T is the first common-risk factor to have been identified in white populations, whereas A419V is now the third risk factor reported to be specific to individuals of Asian ancestry, along with R1628P and G2385R.^{12,14,15} LRRK2 R1628P was not significantly associated with risk in our Asian series. This variant was common only in the Taiwanese series, in which a non-significant protective effect was noted. Our inability to replicate the previously reported risk effect of R1628P is likely to be due to a combination of the low frequency of this variant, natural sampling variation, and population heterogeneity, in view of the results of previous studies of ethnic Han Chinese populations (of note, G2385R did show association).^{14,15}

The identification of a common three-variant haplotype (N551K-R1398H-K1423K) that seems to act in a protective manner (figure 3) is also important. It suggests that the reduced penetrance that is noted in patients with LRRK2-associated parkinsonism might be due to variants acting in cis or trans with the pathogenic variant and that LRRK2 activity can be exploited to modify symptom onset in patients. Any future therapeutic strategies that lower risk in LRRK2-associated parkinsonism might protect against symptomatic onset in idiopathic PD.^{14,27} The previous report¹⁴ of a protective effect with N551K and R1398H showed a reduced kinase

	Aminoacid	White series		Asian series		Arab-Berber series	
		Patients (n=6995)	Controls (n=5595)	Patients (n=1376)	Controls (n=962)	Patients (n=240)	Controls (n=372)
rs2256408	R50H	7 (0.10%)	1 (0.02%)	+	+
rs75054132	A75A	0	1 (0.27%)
rs33995463	L119P	21 (0.31%)	23 (0.44%)	0	2 (0.55%)
rs41286468	L122L	5 (0.08%)	7 (0.13%)
rs112794616	A211V	4 (0.06%)	11 (0.21%)	0	1 (0.27%)
rs56108242	C228S	2 (0.03%)	2 (0.04%)
rs28365216	N238I	3 (0.22%)	2 (0.22%)
rs72546315	H275H	3 (0.04%)	2 (0.04%)	1 (0.43%)	0
rs17490713	N289N	1 (0.01%)	2 (0.04%)	NA	NA
rs41286466	A312A	26 (0.38%)	15 (0.28%)	1 (0.7%)	0	0	4 (1.10%)
rs78501232	E334K	14 (0.21%)	4 (0.07%)
rs113065049	V366M	1 (0.02%)	0
rs34594498	A419V	5 (0.07%)	3 (0.06%)	+	+
rs35847451	S416S	12 (0.18%)	16 (0.29%)
rs75711334	L488L	1 (0.01%)	0
rs79996249	K544E	2 (0.03%)	2 (0.04%)
rs78154388	S663P	2 (0.03%)	2 (0.04%)
rs72546319	V674V	0	2 (0.04%)	0	1 (0.27%)
rs58559150	Q923H	1 (0.01%)	2 (0.04%)	+	+
rs75148313	S973N	1 (0.01%)	2 (0.04%)
rs113217062	I1006M	1 (0.01%)	0
rs76535406	S1096C	0	2 (0.04%)
rs35808389	L1114L	5 (0.07%)	1 (0.02%)
rs74985840	A1151T	1 (0.01%)	5 (0.09%)
rs80179604	S1228T	5 (0.07%)	4 (0.07%)
rs4640000	P1262A	1 (0.01%)	1 (0.02%)	NA	NA
rs72546338	R1325Q	10 (0.15%)	3 (0.06%)	4 (0.29%)	1 (0.11%)
rs17466213	I1371V	7 (0.10%)	4 (0.07%)	1 (0.07%)	0	+	+
rs72546327	T1410M	5 (0.07%)	1 (0.02%)
rs113589830	D1420N	1 (0.01%)	0
rs111435410	A1430A	2 (0.03%)	1 (0.02%)
rs112998035	R1441R	1 (0.07%)	0
rs33939927*	R1441C	10 (0.15%)	0
rs34995376*	R1441H	1 (0.07%)	0
rs74681492	P1446L	10 (0.74%)	6 (0.62%)
rs111501952	V1450I	2 (0.15%)	1 (0.11%)
rs113431708	R1483Q	1 (0.01%)	0
rs35507033	R1514Q	+	+	0	1 (0.27%)
rs33949390	R1628P	7 (0.10%)	0	+	+
rs35303786	M1646T	+	+	3 (1.25%)	2 (0.54%)
rs111503579	L1653L	2 (0.03%)	1 (0.02%)	4 (0.30%)	9 (0.93%)
rs79909111	S1721S	1 (0.02%)	1 (0.02%)
rs263192805	R1728H	1 (0.01%)	3 (0.05%)
rs35602796	M1869T	5 (0.07%)	2 (0.04%)
rs77428810	R1941H	2 (0.03%)	1 (0.02%)
rs34637584*	G2019S	48 (0.71%)	3 (0.06%)	1 (0.07%)	1 (0.11%)	72 (30.25%)	4 (1.10%)
rs111739194	L2063STOP	1 (0.02%)	2 (0.04%)
rs33995883	N2081D	+	+	2 (0.15%)	0	+	+
rs34869625	G2170G	20 (0.30%)	21 (0.39%)	1 (0.60%)	0
rs35658131	Y2189C	1 (0.01%)	2 (0.04%)	+	+
rs113511708	T2356I	7 (0.1%)	5 (0.09%)

(Continues on next page)

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See Online for webappendix

Aminoacid	White series		Asian series		Arab-Berber series	
	Patients (n=6995)	Controls (n=5595)	Patients (n=1376)	Controls (n=962)	Patients (n=240)	Controls (n=372)
(Continued from previous page)						
rs79546190	V2390M	1 (0.01%)	1 (0.02%)
rs78964014	E2395K	1 (0.01%)	0
rs60545352	M2408I	1 (0.01%)	0	..	0	2 (0.54%)

Data are number (%). PD=Parkinson's disease. +=a variant that was noted with a minor allele frequency of at least 0.5% and as such was analysed as a common variant. ..=a variant that was not noted in the series. NA=a variant that was out of the Hardy-Weinberg equilibrium in the specific series. *Pathogenic variants for which the number (%) of carriers is summarised for the entire sample; any carriers of these pathogenic variants were removed from the summaries provided for each of the remaining non-pathogenic variants.

Table 4: *LRRK2* rare variants

activity for the R1398H variant, suggesting this Roc domain substitution might be the most likely functional allele on the haplotype.

Although the results of our study have identified an association of PD only with common variants, they also draw attention to the many rare variants in *LRRK2* that could contribute to disease risk. Genetic loci that contribute to disease risk might do so through variants that span the whole range of minor allele frequencies, from rare mutations to frequent single nucleotide polymorphisms.²⁸ Despite the very large sample size, we noted only three of seven previously described pathogenic *LRRK2* mutations. Hence, the search for mutations contributing to familial PD should include an analysis of single pedigrees, with further assessment in very large population studies. Single pedigrees might result in some false-positive results, which can be filtered out with large population samples. For example, two variants (I1371V and T2356I) have been proposed as pathogenic and to account for the clinical and functional features of *LRRK2*-associated parkinsonism.^{29,30} However, in our study, both variants were noted in patients and controls at the same frequency (table 4). Conversely, we noted other possible rare risk (E334K, R1325Q, and T1410M) and protective (A211V and A1151T) variants; however, because of their low frequency, large meta-analytical approaches are necessary to define their roles fully.

In this study, we focused on exonic variants because all pathogenic variants identified in *LRRK2* so far have been single nucleotide missense changes. However, silent, synonymous variants were also included because they can result in alternative splicing and, since protein translation is a function of codon use and transfer RNA abundance, could affect the rate of protein domain folding and secondary modifications.³¹ Neither copy number variants nor other risk factors in non-coding regions that regulate *LRRK2* expression or alter splicing were assessed in our study.

As new loci for susceptibility to diverse diseases are continuously being discovered in genome-wide association and whole-genome sequencing studies, the results of our study show the importance of revisiting loci at which rare or common variants have been

identified, since they could harbour many more independent signals of genetic risk in different populations.^{25,32,33} Furthermore, *LRRK2* sequencing studies in under-represented populations (eg, from South America, sub-Saharan Africa, Middle East, and western Asia) will undoubtedly show novel ethnic-group-specific risk variants and could clarify the role of variants that were rare or absent in our study. *LRRK2* variants, including novel exonic variants, were reported as part of the 1000 Genome Project, lending support to this hypothesis.³⁴

Large-scale parallel resequencing (targeted genomic capture of the specific regions—eg, gene-specific, exome, transcriptome, and whole-genome sequencing) is likely

Panel: Research in context

Systematic review

We searched PubMed with the terms "*LRRK2*" and "Genetics Parkinson's disease" and identified all *LRRK2* coding variations reported up until April 1, 2010. We also contacted our global network of collaborators and the members of the Genetic Epidemiology Of Parkinson's Disease (PD) Consortium for unreported variants.

Interpretation

By focusing on the role of *LRRK2* variation in PD, we have identified a common risk factor in the white population (M1646T), the third common risk factor in Asian populations (A419V), and a common global protective haplotype (N551K-R1398H-K1423K). This work complements the meta-analysis of PD genome-wide association,²⁵ which suggests a possible association at the *LRRK2* locus. We define some of the genetic variation that is likely to be contributing to the association noted in recent genome-wide association efforts and nominate potential functionally and clinically relevant variants. We show modulation of the underlying toxic effect is possible because of the protective nature of the N551K-R1398H-K1423K haplotype. The identification of common variants that affect risk clearly shows a greater role for *LRRK2* in idiopathic disease than previously thought.

to identify many more variants in candidate genes that might predispose to PD. Characterisation of each variant will require this type of collaborative international effort to define their pathogenicity, frequency in different populations, and contribution to disease pathogenesis through genotype–phenotype assessment.

Contributors

OAR and MJF were the principal investigators and were responsible for the concept and design of the study. AIS-O, JAB, OAR, and CVG were responsible for the technical aspects of the study. MGH and NND were responsible for all the analyses; OAR and MJF were responsible for drafting the report. All authors participated in study design and approach, sample collection, data acquisition, and critical revision and final approval of the report.

Conflicts of interest

JOA, MJF, and ZKW report holding a patent on *LRRK2* genetic variability and MJF has received royalties for licensing of genetically modified *LRRK2* mouse models. DMM declares a patent pending entitled *Methods to treat PD*. CK and RK declare receiving payment in their role as consultants for Centogene and Takeda Pharmaceutical, respectively. All other authors declare that they have no conflicts of interest.

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Caffeine induces apoptosis by enhancement of autophagy via PI3K/Akt/mTOR/p70S6K inhibition

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Key words: apoptosis, autophagy, PI3K/Akt/mTOR/p70S6K, ERK1/2, caffeine

Abbreviations: PI3K, phosphoinositide-3 kinase; 4E-BP1, eukaryotic initiation factor 4-binding protein 1; ERK, extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin; 3-MA, 3-methyladenine; MEFs, mouse embryonic fibroblasts; p70S6K, 70-kDa ribosomal protein S6 kinase; PI, propidium iodide; MPP⁺, 1-methyl-4-phenylpyridinium

Caffeine is one of the most frequently ingested neuroactive compounds. All known mechanisms of apoptosis induced by caffeine act through cell cycle modulation or p53 induction. It is currently unknown whether caffeine-induced apoptosis is associated with other cell death mechanisms, such as autophagy. Herein we show that caffeine increases both the levels of microtubule-associated protein 1 light chain 3-II and the number of autophagosomes, through the use of western blotting, electron microscopy and immunocytochemistry techniques. Phosphorylated p70 ribosomal protein S6 kinase (Thr389), S6 ribosomal protein (Ser235/236), 4E-BP1 (Thr37/46) and Akt (Ser473) were significantly decreased by caffeine. In contrast, ERK1/2 (Thr202/204) was increased by caffeine, suggesting an inhibition of the Akt/mTOR/p70S6K pathway and activation of the ERK1/2 pathway. Although insulin treatment phosphorylated Akt (Ser473) and led to autophagy suppression, the effect of insulin treatment was completely abolished by caffeine addition. Caffeine-induced autophagy was not completely blocked by inhibition of ERK1/2 by U0126. Caffeine induced reduction of mitochondrial membrane potentials and apoptosis in a dose-dependent manner, which was further attenuated by the inhibition of autophagy with 3-methyladenine or *Atg7* siRNA knockdown. Furthermore, there was a reduced number of early apoptotic cells (annexin V positive, propidium iodide negative) among autophagy-deficient mouse embryonic fibroblasts treated with caffeine than in their wild-type counterparts. These results support previous studies on the use of caffeine in the treatment of human tumors and indicate a potential new target in the regulation of apoptosis.

Introduction

Caffeine has a diverse range of pharmacological effects.¹ In addition to its various effects on the cell cycle and growth arrest, higher (4–10 mM) concentrations of caffeine can induce apoptosis in several cell lines, such as 10 mM caffeine in human neuroblastoma cells,² 4 mM caffeine in human pancreatic adenocarcinoma cells³ and 5 mM caffeine in human A549 lung adenocarcinoma cells.⁴ Although caffeine has been reported to modulate cell cycle checkpoints and perturb molecular targets of the cell cycle, the exact mechanism of caffeine-induced apoptosis remains unclear.¹

Autophagy is a key mechanism in various physiopathological processes, including tumorigenesis, development, cell death and survival.^{5,6} It has also been shown to have a complex relationship with apoptosis, especially in tumor cell lines.⁷ Several reports

have shown that autophagy not only enhances caspase-dependent cell death, but is also required for it.⁸ In contrast, it has also been shown that autophagy plays an important role in promoting cell survival against apoptosis.⁷ Caffeine has been reported to inhibit some kinase activities, including various forms of phosphoinositide-3 kinase and mammalian target of rapamycin (mTOR).^{9,10} Recently, in food spoilage studies involving yeast, caffeine has been shown to induce a starvation response,¹¹ which is a key regulator of autophagy causing its induction. However, the exact mechanism by which caffeine induces autophagy is still unknown.

Here we report that higher concentrations of caffeine enhance autophagic flux in a dose-dependent manner in various cell lines. Furthermore, we show that caffeine-induced autophagy is mainly dependent on PI3K/Akt/mTOR/p70S6K signaling and eventually results in apoptosis.

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Results and Discussion

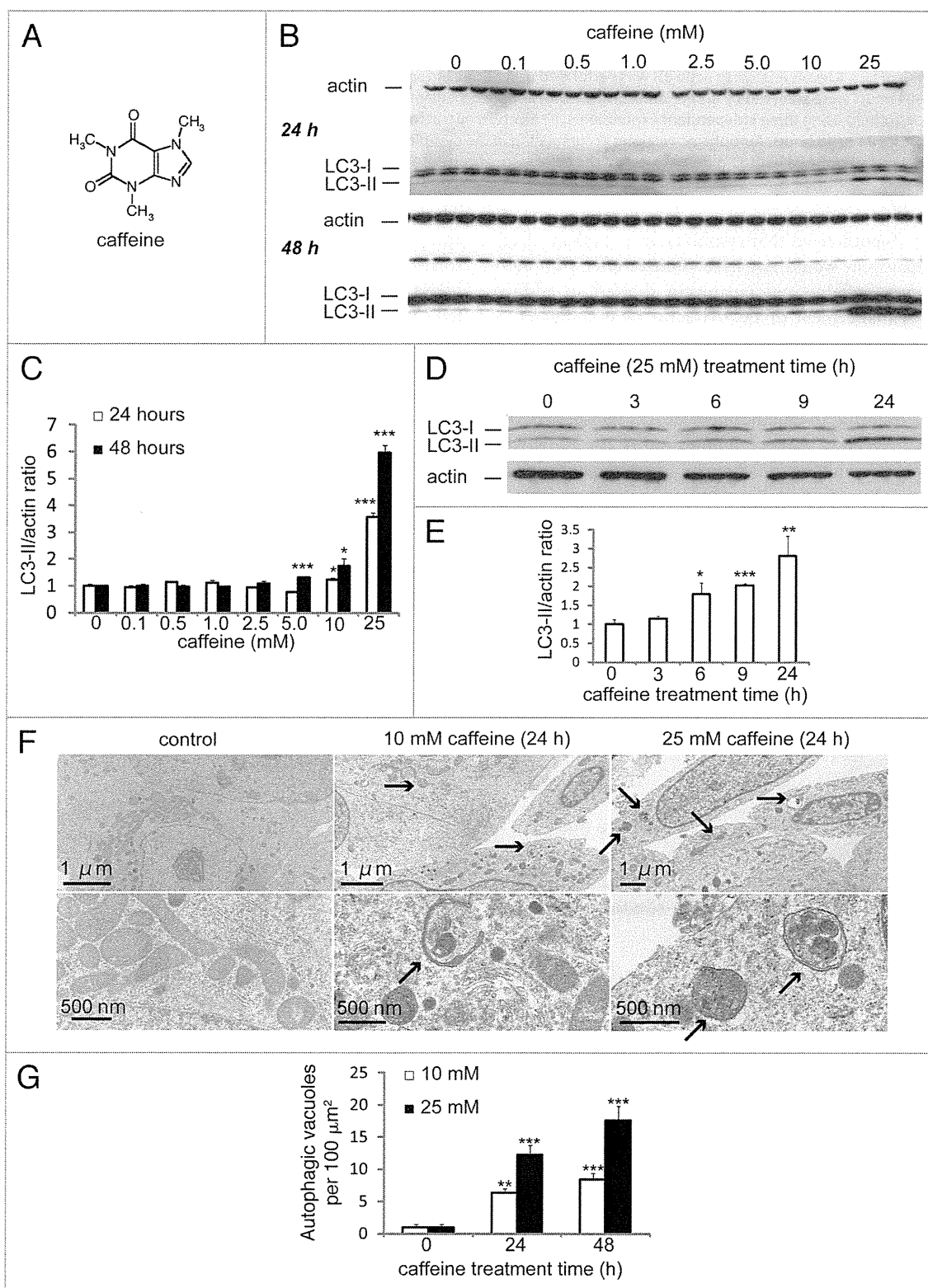
Caffeine (Fig. 1A) is a widely used psychoactive drug that has been used for centuries to increase alertness and energy. It has been reported that caffeine induces autophagy in *Zygosaccharomyces bailii* in association with a starvation response, caused by an unknown mechanism.¹¹ However, it remains unknown whether caffeine affects autophagy in mammalian cells. To determine if caffeine regulates autophagy at a steady state, we first examined levels of the microtubule-associated protein 1 light chain 3 (LC3)-II, which is an LC3-phosphatidyl-ethanolamine conjugate and a promising autophagosomal marker.¹² LC3-II levels (compared to actin loading controls) increased with 5–25 mM caffeine treatment over 48 hours in SH-SY5Y (Fig. 1B and C), PC12D and HeLa cells (Suppl. Fig. S1A and B). The LC3-II/actin ratio also increased in a time-dependent manner in SH-SY5Y (Fig. 1D and E) and HeLa cells (data not shown). Using an electron microscopy technique, the numbers of autophagic vacuoles (AVs) were markedly increased in SH-SY5Y cells treated with 10 or 25 mM caffeine, but not in the control (Fig. 1F and G). Morphometric analysis revealed that the number of AVs per 100 μm^2 of SH-SY5Y cytoplasm in control (Mean \pm standard deviation: 1.3 ± 0.50), whereas that in caffeine-treated cells (10 mM: 8.0 ± 0.82 ; 25 mM: 15 ± 1.9) for 24 hours. Expression levels of p62, a well-known autophagic substrate, were also decreased by caffeine treatment in SH-SY5Y (Fig. 1H and I) and HeLa cells (Suppl. Fig. S1C and D). Furthermore, 10 mM caffeine treatment markedly increased the number of EGFP-LC3-positive vesicles in SH-SY5Y cells transiently transfected with EGFP-LC3 (data not shown) and HeLa cells stably expressing EGFP-LC3 (Figs. 1J and K).^{12,13} This effect was confirmed by the observation that caffeine administration also increased the number of vesicles positive to endogenous LC3 (Suppl. Fig. S1E).

Endogenous LC3 is post-transcriptionally processed into LC3-I, which is found in the cytosol. LC3-I is in turn lipidated to LC3-II, which then associates with autophagosome membranes.¹⁴ LC3-II can accumulate due to increased upstream autophagosome formation or impaired downstream autophagosome-lysosome fusion. To distinguish between these two possibilities, we assayed LC3-II in the presence of E64d plus pepstatin A or bafilomycin A1, which inhibits lysosomal proteases or blocks downstream autophagosome-lysosome fusion and lysosomal proteases, respectively.^{15,16} Caffeine significantly increased LC3-II levels in the presence of E64d plus pepstatin A or bafilomycin compared to E64d plus pepstatin A or bafilomycin alone in (Fig. 2A and B; Suppl. Fig. S1F and G) and HeLa cells (Fig. 2C and D; Suppl. Fig. S1H and I). A saturating dosage of bafilomycin A1 was used in this assay and no further increases in LC3-II levels were observed when cells were treated with higher concentrations. Similar results were observed in PC12D cell lines (data not shown). To confirm the caffeine effect on autophagic flux, we assessed the numbers of autolysosomes and autophagosomes in HeLa cells. The ratio of the numbers of autolysosomes (positive to both LC3 and LAMP2) to autophagosomes (positive to LC3) was increased by 10 mM caffeine treatment for 48 hours (Fig. 2E). Quantification data using ImageJ also showed significant

increase of the ratio (Fig. 2F). These results strongly indicate that high concentration of caffeine treatment enhances autophagic flux.

The class I phosphatidylinositol 3-phosphate kinase (PI3K)/Akt/mTOR/p70ribosomal protein S6 kinase (p70S6K) signaling pathway and the Ras/Raf-1/mitogen-activated protein kinase 1/2 (MEK1/2)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway are two well-known pathways involved in the regulation of autophagy. Both are associated with tumorigenesis and often activated in numerous types of tumors.¹⁷ Therefore, we examined the effect of caffeine on both of these pathways, using western blotting, according to the protocol by Inoki and colleagues.¹⁸ After a 24 hour treatment with caffeine, there was a significant decrease in the levels of phosphorylated p70 S6 kinase, S6 ribosomal protein and 4E-BP1, compared with total normal levels in SH-SY5Y (Fig. 3A), HeLa and PC12D cells (data not shown). Consistent with these results, nonphosphorylated 4E-BP1 proteins were increased by caffeine treatment (Fig. 3A). To further investigate the upstream inhibition of mTOR by caffeine, we examined Ser473 phosphorylation of Akt, which measures both Akt/mTOR and mTORC2 activity. As shown in Figure 3B, treatment with caffeine also decreased the level of phosphorylated Akt in SH-SY5Y cells, which was consistent with a previous report.¹⁹ Similar findings were obtained in HeLa (Suppl. Fig. S2A) and PC12D cells (data not shown). Subsequently, we examined whether caffeine increases the phosphorylation of ERK1/2, a key regulator of autophagy downstream of Akt. As shown in Figure 3C, treatment with caffeine increased phosphorylated ERK1/2. The effects of caffeine on mTOR inhibition were initially detected 3 hours after the addition of caffeine and reached a maximal level after 6 hours in SH-SY5Y (Fig. 3D) and 9 hours in HeLa cells (Suppl. Fig. S2B and C).

Caffeine has been shown to inhibit PI3K and components of the PI3K/Akt pathway.^{9,20} Next, we performed experiments to confirm whether caffeine-induced autophagy is activated through the PI3K/Akt pathway. Insulin or insulin-like growth factor upregulates PI3K and its downstream targets including Akt and mTOR, resulting in the inactivation of autophagy.^{21–23} As shown in Figure 4A and B, insulin treatment for 30 minutes significantly phosphorylated Akt at Ser473, whereas the phosphorylation was completely abolished by additional treatment with caffeine. No significant differences of the LC3-II/actin ratio between caffeine treatment and caffeine treatment with insulin were observed. Also, caffeine and Akt1/2 inhibitors did not have additive effects on the levels of LC3-II/actin ratio compared to the single treatment of caffeine or Akt inhibitors (Fig. 4C and D). To further confirm the caffeine effects on this pathway, cells were transiently transfected with myristoylated Akt (myr-Akt), a constitutively active form of Akt.²⁴ Caffeine treatment of both cells transfected with control vector and myr-Akt markedly decreased the levels of the phosphorylated Akt (Fig. 3E), indicating that caffeine directly inhibits the Akt phosphorylation. If caffeine facilitates autophagy through PI3K/Akt and ERK1/2 signalings, the autophagy should be partially blocked by ERK1/2 inhibition using the mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor, U0126. U0126 significantly but mildly



reversed the levels of LC3-II/actin ratio (Fig. 4F and G). The failure of U1026 to reverse completely the caffeine effect can be explained by the autophagy induction through Akt/mTOR signaling. In addition, only Akt knockdown with inducible short

hairpin RNAs (shRNAs) to specifically and stably knock down all three Akt isoforms sufficiently increases autophagic flux.²⁵ Therefore, we concluded that the caffeine-induced autophagy is mainly dependent on the PI3K/Akt/mTOR pathway.

Figure 1A–G (See opposite page). Caffeine increases autophagic flux in various cell lines. (A) Structural formula of caffeine. (B and C) SH-SY5Y cells treated with various concentrations of caffeine for 24 or 48 hours were analyzed by immunoblotting (B) with antibodies against LC3 and actin. Densitometry analysis of LC3-II levels relative to actin (C) was performed using three independent experiments. (D and E) SH-SY5Y cells treated with 25 mM caffeine for 3–24 hours were analyzed by immunoblotting (D) with antibodies against LC3 and actin. Densitometry analysis of LC3-II levels relative to actin (E) was performed using three independent experiments. (F) Electron microscopic examination of SH-SY5Y cells treated with various concentrations of caffeine for 24 or 48 hours. Autophagic vacuoles accumulating in the cytoplasm are shown by arrows. (G) Morphometric analysis of autophagic vacuoles was performed with 30 different areas of the cytoplasm of control and caffeine-treated cells.

Because caffeine induces autophagy dependently of mTOR inhibition, we hypothesized that combination treatment of caffeine with rapamycin would not have additive effects on autophagy. However, caffeine and rapamycin showed an additive effect on the enhancement of LC3-II/actin ratio compared to the single treatment of caffeine or rapamycin (Fig. 5A and B). Several lines of evidences support the hypothesis that resistance to rapamycin results from a positive feedback loop from mTOR/S6K1 to Akt, resulting in enhancement of Akt phosphorylation at Ser 473.^{26–28} Recently, mutual suppression of the PI3K/Akt/mTOR pathway by combination of rapamycin with perifosine, an Akt inhibitor, induces synergistic effects on autophagy-induced apoptosis as well as enhancement of autophagy, suggesting that dual inhibition of the PI3K/Akt/mTOR by rapamycin with caffeine would be also a rational treatment for cancer.²⁹

Several anti-cancer agents are known to inhibit the PI3K/Akt/mTOR/p70S6K pathway and simultaneously activate ERK1/2, resulting in induction of autophagy in tumor cell lines.^{30,31} The upregulation of this process has beneficial effects in neurodegenerative diseases, such as Parkinson and Huntington diseases, whereas an excess of autophagy can lead to cell death.^{32,33}

Therefore, we decided to investigate whether caffeine-induced autophagy rescues or induces cell death. Using PC12D cells treated with 1-methyl-4-phenylpyridinium (MPP⁺), a well-established Parkinson disease model,³⁴ we determined that 1 mM caffeine treatment was not sufficient for the induction of autophagy (Suppl. Fig. S4 and B) and promoted increased cell viability, whereas >2.5 mM caffeine decreased cell viability (Fig. 6A). In addition, a significant decrease in cell viability was noted in cells treated with >2.5 mM caffeine without MPP⁺. Also, mitochondrial membrane potentials assessed by JC-1 were significantly preserved by 1 mM caffeine treatment compared to the control with MPP⁺, while those were lost by >5 mM caffeine treatment (Fig. 6B and Suppl. Fig. S5A). These data suggest that caffeine-induced autophagy is not protective in these cell lines and leads to cell death.

Autophagy and apoptosis may act independently in parallel pathways or may influence one another.⁷ To confirm the relationship between these pathways in cells treated with caffeine, we examined caffeine effects on the cell cycle with a propidium iodide (PI) staining assay. Treatment with 2.5–10 mM caffeine increased the percentage of cells in the sub-G₁ peak, which is indicative of

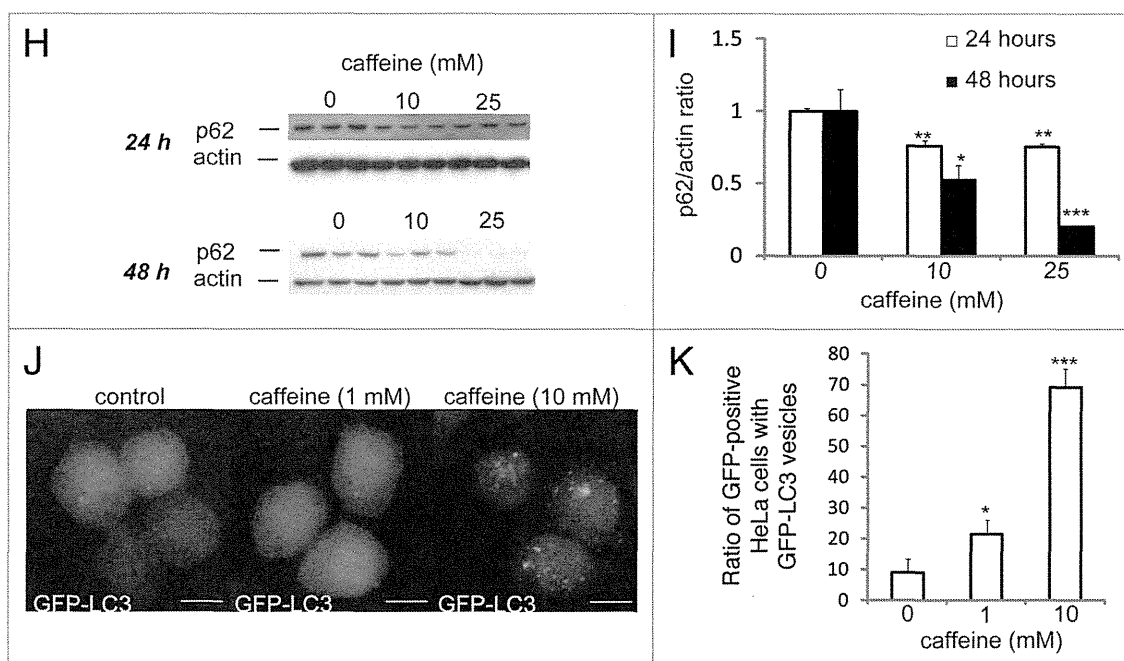


Figure 1H–K. Caffeine increases autophagic flux in various cell lines. (H and I) SH-SY5Y cells treated with various concentrations of caffeine for 24 or 48 hours were analyzed by immunoblotting with antibodies against p62 and actin. Densitometry analysis of p62 levels relative to actin (I) was performed using three independent experiments. (J and K) HeLa cells stably expressing EGFP-LC3 were treated with various concentrations of caffeine for 24 hours and analyzed using confocal microscopy. The percentage of EGFP-positive HeLa cells with >5 EGFP-LC3 vesicles was assessed (K) described previously in reference 43. Error bars, S.D.; **p* < 0.05; ***p* < 0.01.

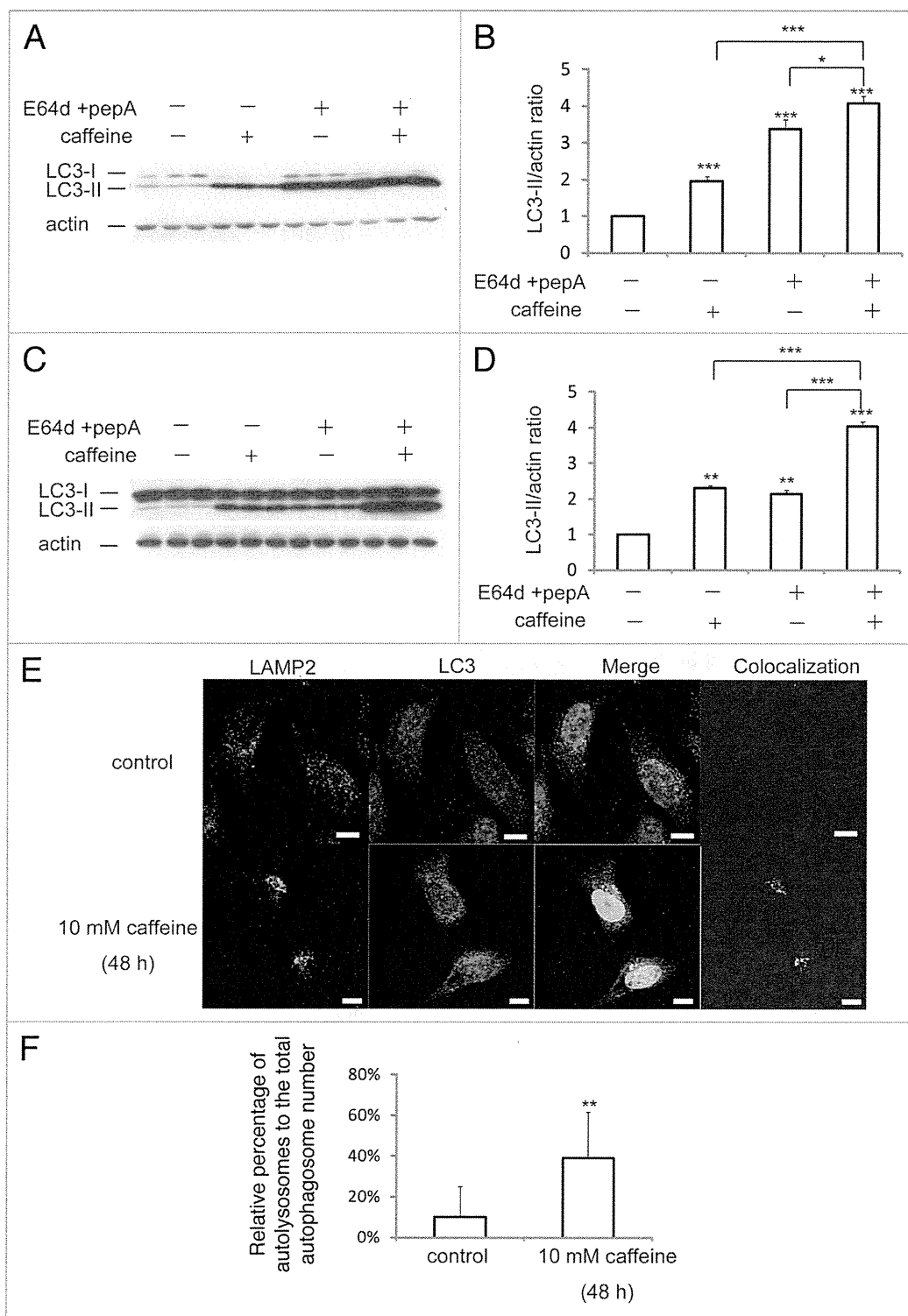


Figure 2. Caffeine does not block autophagosome-lysosome fusion. (A–D) SH-SY5Y (A) or HeLa (C) cells treated with 10 mM caffeine with or without E64d (10 $\mu\text{g}/\text{ml}$) and pepstatin A (10 $\mu\text{g}/\text{ml}$) were analyzed by immunoblotting with antibodies against LC3 and actin. Densitometry analysis of LC3 levels relative to actin in SH-SY5Y (B) and HeLa (D) cells was performed using three independent experiments. (E and F) HeLa cells treated with various concentrations of caffeine for 48 hours were analyzed using confocal microscopy (E). Number of the autolysosomes and autophagosomes were automatically counted using ImageJ “Colocalization” Plugin and the ratios were calculated (F).

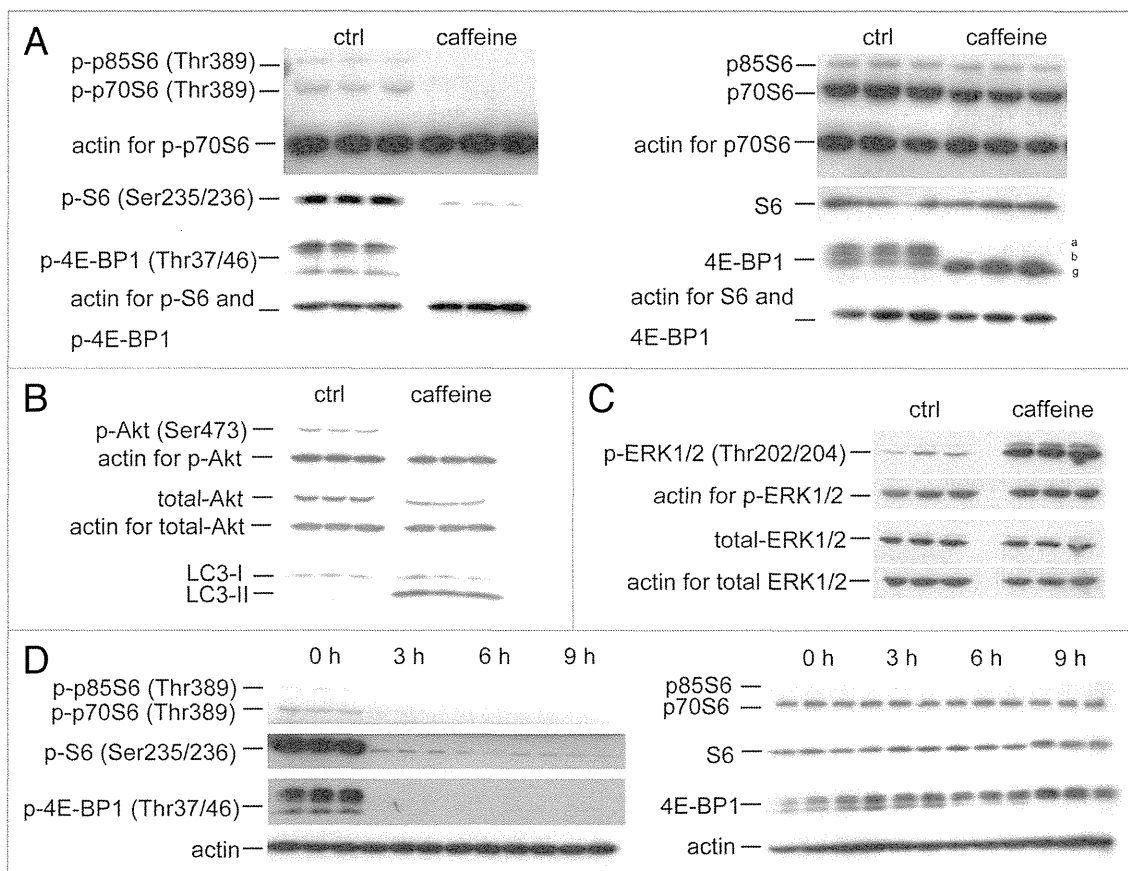


Figure 3. Caffeine inhibits the Akt/mTOR/p70S6 signaling pathway and activates ERK1/2 signaling. (A and B) SH-SY5Y cells treated with or without 10 mM caffeine for 24 hours were analyzed for mTOR activity by immunoblotting for levels of phosphor- and total p70 ribosomal S6 protein, S6, 4E-BP1 (A), Akt (B) and actin. (C) SH-SY5Y cells treated with or without 10 mM caffeine for 0, 3, 6 or 9 hours were analyzed by immunoblotting for levels of phosphor- and total ERK1/2 and actin. (D) SH-SY5Y cells treated with 10 mM caffeine for various time periods were analyzed by immunoblotting for levels of phosphor- and total p70 ribosomal S6 protein, S6, 4E-BP1 and actin.

apoptosis (Fig. 6C). To confirm whether caffeine-induced cell death is apoptotic, we examined the activity of caspase-3, a well-known inducer of apoptosis. Treatment with 10 mM caffeine markedly increased levels of cleaved caspase-3 and decreased full-length caspase-3 in PC12D cells (Fig. 6D), consistent with previous reports on the induction of apoptosis by caffeine.³⁵⁻³⁷

To test whether caffeine-induced apoptosis is dependent on autophagy, we determined whether the inhibition of autophagy by 3-methyladenine (3-MA) or Atg7 siRNA knockdown affects caffeine-induced cytotoxicity in PC12D cells. Treatment with 1 or 5 mM 3MA or Atg7 knockdown significantly decreased the percentage of cell death or cells with reduced mitochondrial membrane potentials caused by caffeine treatment (5 or 10 mM) (Fig. 6E and F and Suppl. Fig. S6B). As can be seen from the increased caffeine-induced apoptosis shown in Figure 6A and C, our data suggests that caffeine-induced autophagy is necessary for apoptotic cell death. To further confirm this, we compared autophagy-deficient mouse embryonic fibroblasts (MEFs), lacking the *Atg7* gene (*Atg7*^{-/-}), without LC3-II expression (Suppl. Fig. S4E), and matched wild-type (*Atg7*^{+/+}) MEFs, in which autophagy is induced by caffeine in a dose-dependent

manner (Suppl. Fig. S4C and D). As expected, the level of caffeine-induced cell death (positive to trypan blue staining) in *Atg7*^{-/-} MEFs was less than that in *Atg7*^{+/+} MEFs (Fig. 7A). The numbers of early apoptotic cells (annexin V positive, PI negative) were significantly increased in both a time-dependent and dose-dependent manner by caffeine treatment of *Atg7*^{+/+} MEFs compared to *Atg7*^{-/-} MEFs (Fig. 7B–D). Also, apoptotic or necrotic cells (annexin V positive) were significantly increased by caffeine treatment of *Atg7*^{+/+} MEFs compared to *Atg7*^{-/-} MEFs (Suppl. Fig. S6). Together, these results indicate that caffeine-induced autophagy partly occurs upstream of apoptosis and is not a protective response to caffeine.

In various tumor cell lines, higher concentrations of caffeine alone induce p53-dependent G₁ phase arrest and under certain conditions apoptosis can also occur in a p53-independent manner.¹ Furthermore, disruption at the G₂/M checkpoint by caffeine allows cells time to repair DNA damage by driving them through mitosis, eventually resulting in apoptosis.^{36,38,39} Consistent with these reports, the results of our study indicate that increased concentrations of caffeine treatment cause a dose-dependent increase in apoptosis. More recently, autophagy, a process long known to

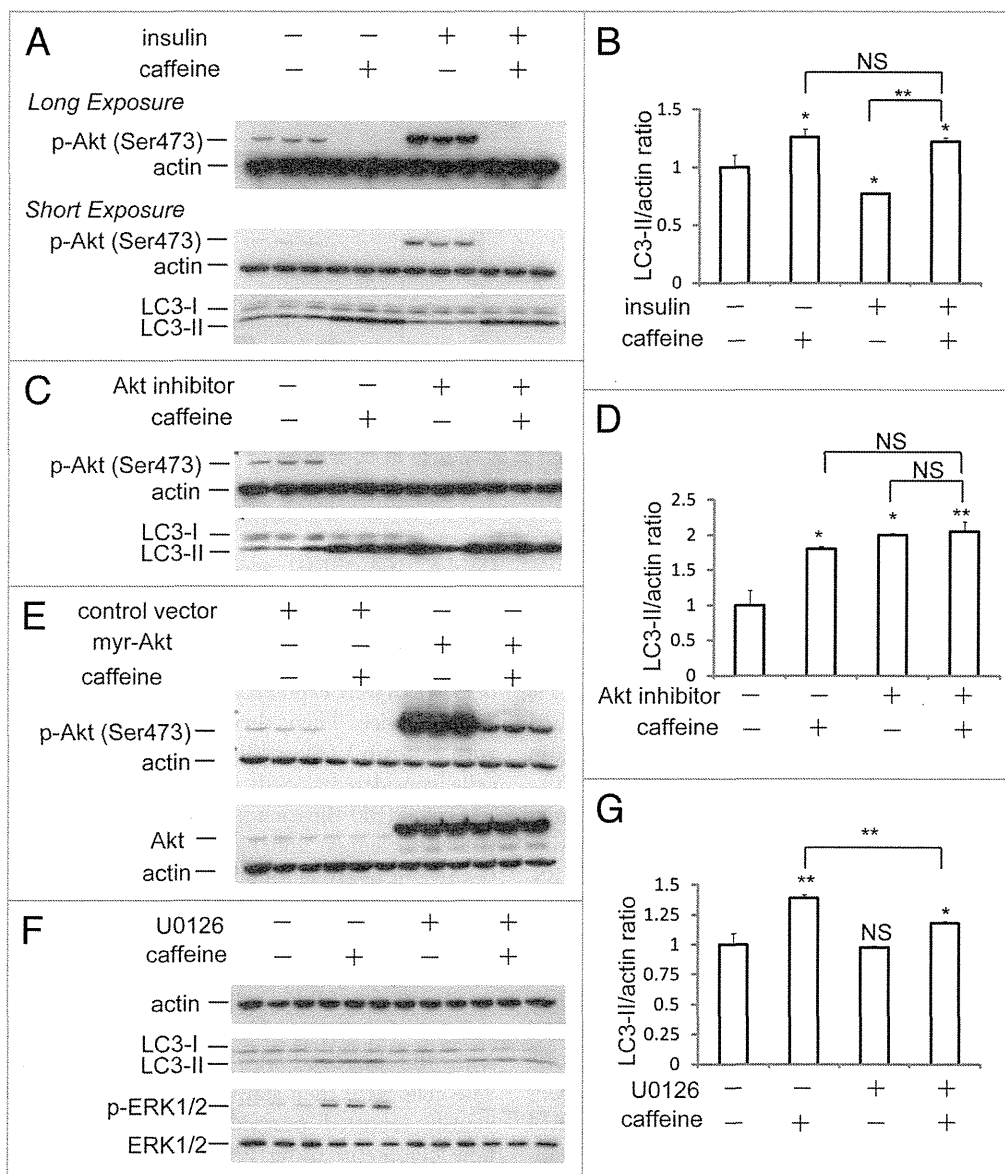


Figure 4. Caffeine-induced autophagy is dependent on PI3K/Akt/mTOR pathway. (A) SH-SY5Y cells treated with 25 mM caffeine for 3 hours followed by treatment with or without 200 nM insulin for 30 minutes were analyzed by immunoblotting. (B) Densitometry analysis of LC3-II levels relative to actin was performed using three independent experiments. (C) SH-SY5Y cells treated with 25 mM caffeine, 50 μ M Akt1/2 inhibitors or 25 mM caffeine with 50 μ M Akt1/2 inhibitors for 6 hours were analyzed by immunoblotting. (D) Densitometry analysis of LC3-II levels relative to actin was performed using three independent experiments. (E) SH-SY5Y cells were transfected for 24 hours with either a control plasmid DNA (pcDNA3.1) or a plasmid encoding constitutively active Akt (myr-Akt), and then treated with H₂O or 10 mM caffeine for 6 hours. Immunoblotting was performed using antibodies against Akt, p-Akt (Ser 473) and actin. (F) SH-SY5Y cells treated with 25 mM caffeine with or without 20 μ M U0126 for 6 hours were analyzed by immunoblotting using antibodies against actin, LC3, p-ERK and ERK. (G) Densitometry analysis was performed using three independent experiments. Error bars, SD; *p < 0.05; **p < 0.01; N.S., not significant.

provide a survival advantage to cells undergoing nutrient deprivation and other stresses, has also been linked to the cell death process.⁷ The cross-talk between apoptosis and autophagy is complex and sometimes contradictory; however, it is critical to the overall fate of the cell. In this study, we have shown that autophagy is induced by higher concentrations of caffeine without starvation, mainly via the inhibition of PI3K/Akt/mTOR/p70S6K signaling. Likewise, when caffeine-induced autophagy is blocked by 3-MA

treatment or *Atg7* knockout, apoptosis is partially attenuated, suggesting that caffeine-induced autophagy occurs upstream of caffeine-induced apoptosis. It also indicates the involvement of other pathways in caffeine-induced apoptosis. These results provide new insight into the effects of caffeine on cell death and survival and its use as a possible intervention strategy for the upregulation of apoptosis by a harnessing of its autophagic activity in tumor treatment.