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Bleomycin hydrolase is associated with risk of sporadic Alzheimer's disease

Genetic studies of Alzheimer's disease (AD) have revealed causative mutations in the amyloid precursor protein (*APP*), presenilin 1(*PSEN1*) and presenilin 2 (*PSEN2*) genes in familial AD[1] and identified apolipoprotein E (*APOE*) as an AD susceptibility locus in sporadic disease[2]. These genetic factors may facilitate deposition of intracortical amyloid plaques, believed to be an early and necessary event in AD pathogenesis[3]. Papain superfamily cysteine proteases, resembling human bleomycin hydrolase, have been implicated in formation of amyloidogenic peptides through APP cleavage[4,5]. We have found the gene for bleomycin hydrolase (*BH-PEN*) to be a novel susceptibility locus for development of AD in an association study comprising two independent patient populations.

A total of 357 AD cases and 320 controls were obtained from the University of Pittsburgh Alzheimer's Disease Research Center (ADRC) and the Indiana Alzheimer Disease Center Cell Repository (IADC). *BH-PEN* was genotyped for the A1450G polymorphism that results in an I443V conserved amino acid substitution in the carboxy terminus of the protein[6] using PCR-SSCP, as previously described[7]. *BH-PEN* A1450 and G1450 allele frequencies in pooled AD cases and controls were statistically indistinguishable. The G/G homozygote genotype distribution, however, was significantly different between AD cases (12.7%) and controls (6.6%; $P<0.001$) (Table 1). Significant differences were not seen in the A/A homozygote and A/G heterozygote genotype distributions. The odds ratio for the homozygote G/G compared to A/A and A/G was 2.05 ($P=0.009$, 95% CI=1.20–3.53).

Table 1 • *BH-PEN* allele frequency and genotype distribution

	Total	Allele frequencies		Genotype frequencies		
		A	G	A/A	A/G	G/G
ARDC clinical cases	131	0.70 ± 0.03	0.30 ± 0.05	0.489	0.412	0.100
ADRC confirmed cases	151	0.67 ± 0.03	0.33 ± 0.05	0.426	0.358	0.152
IADC confirmed cases	75	0.65 ± 0.05	0.35 ± 0.07	0.426	0.440	0.133
AD cases[a]	357	0.68 ± 0.02	0.33 ± 0.03	0.476	0.398	0.127
ADRC controls	124	0.70 ± 0.04	0.30 ± 0.05	0.468	0.460	0.073
IADC controls	57	0.69 ± 0.05	0.30 ± 0.08	0.456	0.474	0.070
Pittsburgh controls[b]	139	0.73 ± 0.03	0.27 ± 0.05	0.511	0.432	0.058
AD controls[a]	320	0.71 ± 0.02	0.29 ± 0.04	0.484	0.450	0.066

[a]Pooled population. [b]Random population sample from the Pittsburgh geographical area.

To determine if the influence of *BH-PEN* is independent of the *APOEε4* risk factor, we stratified AD cases and controls according to *APOE* genotype (*ε2*, *ε3*, *ε4* alleles). Surprisingly, the frequency of the G/G homozygote was significantly higher in AD cases than controls only in the non-*APOEε4* group (15.9% in cases *versus* 4.7% in controls; $P<0.001$) (Table 2). The odds ratio for developing AD with the *BH* G/G genotype in the absence of an *APOEε4* allele was 3.81 ($P=0.0007$, 95% CI=1.77–8.26). In the presence of an *APOEε4* allele, the odds ratio was 0.98. Therefore, the increased risk derived from the G/G genotype was observed only in the absence of the *APOEε4* allele.

Logistic regression analysis of pooled data was performed modelling the risk of AD for each *BH-PEN* genotype and adjusting for age, gender, *APOE* genotype and 1-antichymotrypsin (*ACT*) genotype (BMDP Statistical Software Inc.). The *BH-PEN* G/G genotype conferred an approximately two-fold risk for AD compared with the A/A genotype (OR=2.10, $P<0.05$). No increased risk was observed for the heterozygote A/G consistent with a recessive model of inheritance of risk. In those individuals lacking an *APOEε4* allele, the *BH-PEN* G/G genotype substantially increased the risk of development of AD (OR=4.00, $P<0.05$). The *APOEε4* risk was not modified by the interaction between the *BH-PEN* genotype G/G and *APOEε4*. *ACT*, a modifier of the AD risk associated with the *APOEε4* allele[8], also acted independently of *BH-PEN*.

Bleomycin hydrolase is highly conserved through evolution; however, the only known activity of the enzyme is metabolic inactivation of the chemotherapeutic glycopeptide bleomycin. *BH-PEN* is thus a primary candidate gene for protection against potentially fatal bleomycin-induced pulmonary fibrosis and bleomycin resistance in tumours[9]. *BH-PEN* has been cloned and encodes a 455 amino-acid protein containing the signature active site residues of the cysteine protease papain superfamily[6,10] and aminopeptidase activity that is blocked by the irreversible cysteine protease inhibitor E-64. Both yeast and human BH also possess endopeptidase activity[11,12]. Human *BH-PEN* is encoded by a single-copy gene located at 17q11.2 (refs 7,13).

Yeast BH is a bi-functional protein with DNA binding and protease activity and is a member of the galactose regulon[14,15]. The yeast BH crystal structure reveals a homohexameric structure with a prominent central channel housing the active sites resembling the 20S proteasome and suggests the C-terminal domain is a key regulatory region[14]. Minimal deletions in this region appear to affect both aminopeptidase and endopeptidase activity[11,12]. Similarly, deletion of the C-terminal eighteen amino acids of human BH, which includes the polymorphic residue 443, abolished enzymatic activity[11]. Given the location of the polymorphism in the C-terminal domain of BH, the A1450G substitution may affect BH activity *in vivo*. Alternatively, the conservative I443V sub-

Table 2 • *BH* genotype frequencies stratified by *APOE*

		Totals	Genotype frequency A/A	A/G	G/G
APOE ε2/ε2, ε2/e3, ε3/ε3	Cases	126	0.492	0.349	0.159
	Controls	233	0.489	0.464	0.047
APOE ε2/ε4, ε3/e4, ε4/ε4[a]	Cases	231	0.468	0.420	0.112
	Controls	87	0.471	0.414	0.115

[a]The *APOEε4* allele frequencies were 0.39±0.03 for AD cases and 0.14±0.03 for controls.

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stitution may not influence enzymatic activity[6] and the A1450G polymorphic site may simply be in linkage disequilibrium with another locus that is functionally significant in AD pathogenesis. *BH-PEN* is the first susceptibility locus for AD whose impact on risk is confined to individuals lacking an *APOEε4* allele.

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Escape from X inactivation of *Smcx* is preceded by silencing during mouse development

The majority of genes on the inactive mammalian X chromosome are silenced, but a small number of genes escape X inactivation[1]. Haploinsufficiency for such genes has been implicated in the aetiology of monosomy X, or Turner syndrome, which results in poor viability *in utero*[2]. To investigate the developmental controls of escape from X inactivation, including potential differences between individual cells, expression levels of a gene known to escape X inactivation, *Smcx* (selected mouse cDNA on the X; ref 3,4), were measured from the active and inactive X chromosomes in single cells from embryos and adult female mice from C57BL/6Ros (B)×*M.spretus* (S) crosses. RT-PCR products for *Smcx* and for *Rps4* (ribosomal protein S4), a control gene subject to X inactivation[5,6], were obtained in 54% of 198 cells, suggesting a comparable efficiency of amplification for both genes. The proportion of cells with successful amplification of *Smcx* and *Rps4* remained constant throughout development and in adult tissues, confirming ubiquitous *Smcx* expression (data not shown).

SNuPE (single nucleotide primer extension) assays[7] were carried out to quantify allele-specific expression of *Smcx* and *Rps4* within each cell (Fig. 1*a*). A linear relationship was observed between ratios of allelic expression and input parental RNA or cDNA ratios, ranging from 1/1 to 1/100. A single expressed *Rps4* allele (S or B) in each cell indicated that X inactivation was maintained from 6.5 dpc throughout development and adulthood as expected. (Preferential inactivation of the paternal S allele is likely due to different *Xce* alleles in the two mouse species.) Extraembryonic membranes with paternal X inactivation[8] were removed prior to analysis, although the presence of cells of extraembryonic origin can not be completely ruled out at 6.5 dpc.

Smcx already escaped X inactivation in a majority of cells from 6.5 dpc embryos, as shown by its biallelic expression (Fig. 1*a*). However, *Smcx* expression levels were generally lower from the inactive X, compared with the active X. Further, *Smcx* was completely inactivated in some cells (12% of 25 cells), with no detectable signal from the allele on the inactive X (Fig. 1*a*,*b*). Such cells persisted as 15% of a total of 42 cells at 8.5, 11.5 and 13.5 dpc. Cells with apparently complete inactivation of *Smcx* were not the result of maternal cell contamination, because PCR of genomic data yielded both B and S alleles as expected in F1 embryos (Fig. 1*b*). After quantification, *Smcx* expression from the inactive X ranged from 0% (representing complete inactivation) to approximately 50% (representing complete escape) of the total, with the greatest cell-to-cell variability at 6.5 dpc, shortly after X inactivation is known to occur (Fig. 2).

In adult tissues, *Smcx* allelic expression was much less variable than in the embryo and showed nearly equal expression from both X chromosomes (Fig. 2). *Smcx* expression from the inactive X in 40 adult cells had a mean value of 48% ± 1.1 of the total. A control autosomal gene, *Clc3* (ref. 9), showed a mean BL/6 expression of

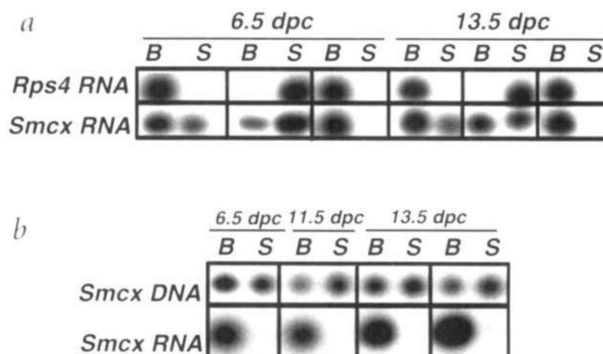

Fig. 1 Single-cell SNuPE analysis. Primers stop one nucleotide 5′ of the allelic mismatch in BL/6×*M. spretus* F1 female 6.5- and 13.5-dpc embryos (sexed by *Sry* PCR). ***a***, Expression of either the B or S allele of *Rps4* marks the active X. Expression of *Smcx* alleles either equal or with lower or no expression from the allele on the inactive X. ***b***, Control DNA analysis shows both *Smcx* alleles present in all cells.