

A twofold increase in *BRCA* mutation related prostate cancer among Ashkenazi Israelis is not associated with distinctive histopathology

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The magnitude of the risk of prostate cancer among Ashkenazi Jewish carriers of the common mutations in the *BRCA1* and *BRCA2* genes, the so-called Ashkenazi *BRCA* founder mutations (*BRCA1* 185delAG and 5382insC, and *BRCA2* 6174delT), remains uncertain. Two large epidemiological studies have suggested that the cumulative incidence of prostate cancer among male first degree relatives of individuals with a founder mutation may approach 30% by 80 years of age.^{1,2} Smaller clinical studies have, in general, not demonstrated an increased frequency of founder mutations among Jewish men with prostate cancer.^{3–6} Only one study to date has examined clinical characteristics of prostate cancer patients with founder mutations.³ In this study, the three *BRCA* associated tumours that were identified appeared to be associated with biologically more aggressive disease, presenting with higher PSA levels, higher Gleason scores and more advanced stage at diagnosis, than did prostate cancers diagnosed in men without mutations.

Founder *BRCA* mutations occur in more than 2% of Ashkenazi Jews.^{1,7,8} Thus, an increased risk of prostate cancer among *BRCA* carriers, or an aggressive phenotype in *BRCA* associated prostate cancers, could constitute a significant cancer burden in this group, and would have substantial management implications for male carriers of Ashkenazi founder mutations.

We undertook the current study to obtain a more stable estimate of the Ashkenazi founder mutation rate among Jewish men diagnosed with prostate cancer, and to determine if prostate cancer occurring in carriers of the common Ashkenazi *BRCA1* or *BRCA2* mutations is associated with distinctive pathological or clinical features. The precedent for this hypothesis is best illustrated by the hereditary renal cancer syndromes, in which recognising specific histological subsets of subjects has facilitated the identification of genetically distinct disorders.^{9,10}

All men newly diagnosed with prostate cancer were identified at sixteen of the 23 hospital based and reference pathology laboratories that provided histological diagnosis of prostate cancer in Israel from 1994 to 1995. This time period was selected because it antedated the widespread use of prostate specific antigen (PSA) screening in Israel. One laboratory declined participation, and a second had no Ashkenazi cases during the period of interest. To represent the remaining five small laboratories, a larger centre, which drew its cases from the same geographic region, was selected for study. Only half of the cases diagnosed at one large reference laboratory were enrolled, by selecting all cases diagnosed at that facility every other month. Each pathology report was reviewed to verify the diagnosis of prostate cancer,

Key points

- To estimate the risk of prostate cancer (PrC) associated with the common Ashkenazi founder mutations of the *BRCA1* and *BRCA2* genes, and to investigate the histopathology characteristics of mutation related PrC, we identified 979 Ashkenazi Jewish men diagnosed with PrC during 1994–1995 in 16 Israeli hospitals and reference laboratories.
- DNA obtained from paraffin sections was tested for the three founder mutations in 940 (96%) cases, and a mutation was identified in 30/940 (3.2%), a two fold increase compared with a referent group of Ashkenazi men over 50 years old with no history of prostate cancer (21 of 1344 (1.6%); OR = 2.1; 95% CI = 1.2–3.6).
- In the entire cohort, no difference was found in the mean age at diagnosis between cases with and without a founder mutation.
- A representative histology slide was blindly reviewed from 29/30 (97%) of Israeli *BRCA* associated PrC cases and 145 individually matched non-carrier PrC cases.
- No differences were noted between the two sets of cases regarding: the mean Gleason score; presence of prostatic intraepithelial neoplasia or atypical adenomatous hyperplasia; or other histopathology features.
- These results are consistent with a doubling of the risk of PrC among carriers of an Ashkenazi *BRCA* founder mutation, and do not suggest that mutation related PrC is characterised by early age at diagnosis or distinct histopathology features.

and to exclude cases with a previous diagnosis of prostate cancer.

All identified prostate cancer subjects were matched to the Israeli Population Registry. Patients were considered to be of Ashkenazi heritage if they were born in Europe or the United States or, if Israeli born, if both parents were born in Europe or the United States. The diagnostic pathology block was retrieved from Ashkenazi prostate cancer cases when available. Minimal information on cases was retained prior to sample anonymisation including: age at diagnosis, hospital of registration, tissue acquisition method (core biopsy, prostaticectomy, or transurethral resection of the prostate).

Abbreviations: PSA, prostate specific antigen

Participants were then assigned a unique study identifier linked to the pathology specimens. The pathology material was anonymised prior to pathology review and data analysis. Due to the anonymous nature of the study design, medical records were not abstracted, and no information concerning stage or PSA levels at the time of diagnosis was obtained. The study was done under a waiver of the requirement for Institutional Review Board review granted by the National Institutes of Health Office of Human Subjects Research.

We estimated the population founder mutation carrier frequency in Ashkenazi men over 50 years old using data from two study populations: (a) Ashkenazi Jewish male volunteers with no personal history of prostate cancer in the Washington Ashkenazi Study (WAS) (Struewing *et al*¹ and J Struewing, unpublished data), and (b) Israeli men enrolled in the Molecular Epidemiology of Colorectal Cancer (MECC) study, an Israeli case-control study with phenotype and genotype data available for 901 population based controls (Gruber *et al*¹¹ and S Gruber and G Rennert, unpublished data). Controls were individually matched by sex, year of birth, and clinic to incident cases of colorectal cancer diagnosed in northern Israel between March 1998 and December 2002.¹¹

For the pathology review, each prostate cancer case identified as positive for one of the three Ashkenazi founder mutations was matched to five non-carrier prostate cancer cases by tissue acquisition procedure (core needle biopsy, transurethral resection of the prostate, or prostatectomy). The best available match for each carrier case was made using a four tiered system: (a) an exact match by age and pathology laboratory; (b) the closest age match (within 5 years) from the same pathology laboratory; (c) the closest age match (within 5 years) from the same type of laboratory (hospital based or referral laboratory); or (d) the closest age match (within 3 years) from any laboratory type. To select five controls per case, we first selected one control per case, selecting a control from the most optimal tier. A second control for each case was then selected, and so forth. Controls were selected without replacement. Thus, once a control was selected as a match for a case, it was unavailable for selection for another case. Histology slides from carrier and non-carrier cases were randomly arranged, and reviewed by a single pathologist (PD), who was blinded to the mutation status of the cases.

Unstained 5 µm paraffin sections were treated with xylene, rehydrated, and digested with proteinase K, followed by phenol extraction and ethanol precipitation. Using 2–4 µl of the sample, a multiplex PCR was performed to detect the three founder mutations, as described previously.¹²

Mutation rates were estimated as simple proportions. These rates were compared with the expected rates in controls using the χ^2 test with Yates' continuity correction. An extended McNemar test was employed to compare binary outcomes in mutation related cases with their matched controls. Here, k_i = number of controls in matched set i , x_i = case result (0 or 1) in matched set i , m_i = number of controls in matched set i with positive result (equal to 1) and n = number of matched sets (equal to 27 in our study). The estimate of the odds ratio was obtained from the sum of $x_i(k_i - m_i)$ over each matched set divided by the sum of $m_i(1 - x_i)$. To test whether the odds ratio was different from 1, for each matched set the observed minus the expected value of x_i , ($O_i - E_i$) was equal to

$$x_i - (m + x_i)/(k_i + 1),$$

and its variance, V_i was equal to

$$(m_i + x_i)(k_i + 1 - m_i - x_i)/(k_i + 1)^2.$$

The sum of $O_i - E_i$ over all matched sets was denoted by $O - E$, and the sum of V_i over all matched sets by V . The test statistic was then calculated as $(O - E)^2/V$. Because of the small sample size in the case-control study, the usual χ^2 approximation did not provide a reliable reference distribution for this statistic under the null hypothesis. We therefore estimated the p value and confidence limits for the odds ratio by Monte Carlo simulation, using runs of 10 000 simulations.

To compare ordered outcomes, such as Gleason's grade, between carriers and their matched controls, the stratified Wilcoxon test was used as the test statistic¹³ and, again, the p values and confidence limits for the difference in mean tumor grade were estimated from runs of 10 000 Monte Carlo simulations. A three way test for the difference in the least squared mean age at diagnosis was performed among *BRCA1* positive, *BRCA2* positive and mutation negative men, using SAS PROC GLM (SAS Institute Inc., Carry, NC, USA). Associations with $p < 0.05$ were considered to be significant.

Among Ashkenazi men, 992 eligible cases of prostate cancer were identified, representing approximately two thirds of all prostate cancer cases diagnosed in Israel during the study period. In 13 cases (1%), no pathology material could be retrieved. In the remaining 979 cases, a haematoxylin and eosin stained slide was obtained for review, and five unstained 5 µm paraffin fixed sections were obtained as the DNA source for mutation testing. DNA was extracted and mutation testing was successfully completed for all three of the founder *BRCA* mutations on 940 (96%) of these cases.

In the entire cohort, although the *BRCA2* related cases were slightly younger at diagnosis than the non-carriers and the *BRCA1* related cases, there were no statistical differences in the mean ages of these three groups (*BRCA2* 71.6 years, non-carriers 73.6 years, and *BRCA1* 74.2 years). One prostate cancer case, a non-carrier, was diagnosed at the age of 49 years. All other cases were diagnosed at 50 years of age or later. Among the prostate cancer cases, mutation carriers were more frequently diagnosed by core needle biopsy (29/30 (93%) v 625/910 (69%)) than non-carriers. This difference in the mode of diagnostic tissue acquisition was significantly different between mutation carriers and non-carriers ($\chi^2 = 9.03$, $p = .01$).

The overall founder mutation prevalence among Israeli prostate cancer cases was 30/940 (3.2%). Of these, 16 (1.7%) had a *BRCA1* mutation [185delAG, 14 (1.5%); 5382insC, 2 (0.2%)] and 14 (1.5%) had a *BRCA2* 6174delT mutation. In the WAS and MECC studies, the mutation frequencies found among Ashkenazi men over the age of 50 years with no history of prostate cancer were 1.49 and 1.69 respectively (table 1). The difference between these two frequencies was not statistically significant (OR = 0.88; 95%CI = 0.3–2.3). When the two comparison groups were combined (WAS+MECC), the increased frequency of any of the founder mutations and of the *BRCA1* 185delAG founder mutation among Israeli PrC cases reached statistical significance (table 1).

Cases and controls in the pathology review were well matched. An exact match was found for 85% of the first selected controls and for 62%, 54%, 50% and 39% of the second to fifth controls, respectively. In only four cases was a fourth tier control selected for inclusion. One of the 30 identified mutation carriers (with a *BRCA1* 185delAG mutation) and his five matched controls were eliminated from the pathology case-control study, because the reviewing pathologist was not able to confirm the diagnosis of prostate cancer based on the limited material available for review. The remaining 29 confirmed prostate cancer cases with an identified *BRCA1* or *BRCA2* founder mutation, and a total of 145 individually matched non-carrier cases constituted the pathology review group.

Table 1 The frequency of *BRCA1/2* Ashkenazi founder mutations among Israeli Ashkenazi men diagnosed with incident prostate cancer 1994–1995, and among men aged 50 years and over with no history of prostate cancer in the Washington Ashkenazi Study (WAS), and in the Molecular Epidemiology of Colorectal Cancer (MECC) study

	<i>BRCA1</i> (n (%))		<i>BRCA2</i> (n (%))		Total positive	Total (n)
	185delAG	5382insC	6174delT			
Israeli Prostate Study	14 (1.49)	2 (0.21)	14 (1.49)		30 (3.19)	940
WAS*	6 (0.7)	2 (0.2)	5 (0.6)		13 (1.49)	872
OR	2.18	0.93	2.62		2.18†	
95% CI	0.78 to 6.96	0.07 to 12.82	0.89 to 9.34		1.09 to 4.43	
Israeli Prostate Study	14 (1.49)	2 (0.21)	14 (1.49)		30 (3.19)	940
MECC*	2 (0.42)	1 (0.21)	5 (1.06)		8 (1.69)	472
OR	3.55	1.00	1.41		1.91	
95% CI	0.80 to 15.70	0.09 to 11.1	0.50 to 3.94		0.87 to 4.20	
Israeli Prostate Study	14 (1.49)	2 (0.21)	14 (1.49)		30 (3.19)	940
WAS+MECC*	8 (0.59)	3 (0.22)	10 (0.74)		21 (1.56)	1344
OR	2.52	0.22	2.02		2.08†	
95% CI	1.05 to 6.04	0.16 to 5.72	0.16 to 5.72		0.16 to 5.72	

*Ashkenazi Jewish male controls 50 years and above without history of prostate cancer.

†p<0.05.

Mutation carriers and non-carriers were comparable with respect to all the histological characteristics evaluated. While prostate cancer among carriers was more likely to arise in the setting of chronic prostatitis and to be associated with neuroendocrine features than was prostate cancer in non-carriers, these findings were not statistically significant. No significant differences were noted in either the mean primary or secondary Gleason grades or in the Gleason sum (table 2).

A number of case series have examined the carrier frequency among Ashkenazi men with prostate cancer^{4–6} (table 3). While the numbers of mutation carriers included in these studies have been very small, these studies have not suggested a large increase in prostate cancer risk associated with founder mutations. One small case-control study of Israeli prostate cancer cases and matched controls did not suggest an increased carrier frequency among cases, but

provide a suggestion of a more aggressive phenotype among mutation carriers.

Two larger studies have estimated risk of prostate cancer indirectly, based on the history of prostate cancer in relatives of founder mutation carriers and non-carriers.^{1,2} Struwing and colleagues used a kin cohort analytic approach to estimate the cumulative incidence of cancer associated with Ashkenazi founder mutations in a study of over 5000 American Ashkenazi Jewish volunteers from the Washington DC area [the Washington Ashkenazi Study (WAS)].¹ In this study, the founder mutations were found at a similar frequency to that reported in previous studies of Ashkenazi Jews. The risk of prostate cancer by 70 years of age was estimated to be 16% (95% CI 4%–30%) among male carriers of any founder mutation, compared with 3.8% among non-carriers (95% CI 3.3%–4.4%), a risk equal to that of

Table 2 Pathological features of Israeli prostate cancer cases by carrier status

Pathology characteristic	Carriers		Non-carriers		OR	95% CI	p
	n = 29	(%)	n = 145	(%)			
Benign and pre-neoplastic features							
Atrophy	3	10	11	8	1.40	0.32–5.0	0.72
Benign prostatic hyperplasia	5	16	19	13	1.43	0.45–4.2	0.76
Chronic prostatitis	9	31	23	16	2.47	0.93–6.4	0.06
Basal cell hyperplasia	1	3	10	7	0.44	0.02–3.3	0.70
Atypical adenomatous hyperplasia	0	0	2	1	*	—	—
Atypical small acini proliferation	1	3	9	6	0.56	0.02–4.1	0.69
Prostatic intraepithelial neoplasia							
Low grade	1	3	17	12	0.29	0.01–1.78	0.32
High grade	3	10	22	15	0.67	0.15–2.37	0.57
Low or high grade	4	14	38	26	0.49	0.13–1.39	0.23
Adverse pathological features							
Perineural invasion	16	55	77	53	1.08	0.48–2.47	1.00
Lymphovascular invasion	0	0	5	3	0	0.00–4.8	0.59
Neuroendocrine features	6	21	13	9	2.7	0.88–8.1	0.09
Extra prostatic extension	3	10	6	4	†	—	—
Seminal vesicle involvement	1	3	2	1	†	—	—
Any adverse feature	21	72	95	66	1.40	0.54–3.5	0.51
Mean Gleason Score	7.1	—	7.0	—	—	—	0.70

*Numbers too small for estimation of odds ratio

†In the matched analysis, only five sets had both a case and at least one control that could be evaluated. Two of the cases, and none of the controls, had extra prostatic extension.

‡In the matched analysis, only one set had both a case and a control that could be evaluated. The case had seminal vesicle involvement, but the control did not.

Table 3 Risk of prostate cancer associated with *BRCA1/2* Ashkenazi Jewish (AJ) founder mutations (literature review)

Study	Population	n	Number (%) of individual subjects tested			Outcomes
			BRCA1		BRCA2	
			185delAG	5382insC	6174delT	
Kin cohort Struewing <i>et al</i> ¹	N. American AJ volunteers; mutation carriers and non-carriers	5318	41 (0.8)	20 (0.4)	59 (1.2)	Risk of prostate cancer by 60 and 80 years of age among first degree relatives of volunteers: with mutations 16%, 39%; without mutations 1%, <10%, respectively Risk of prostate cancer by the age of 85 years among first degree relatives of Jewish breast cancer cases: with mutations 34%; without mutations 13%
Foulkes <i>et al</i> ²	N. American AJ women with breast cancer	412	26 (6.3)	8 (1.9)	15 (3.6)	
Case-control Hubert <i>et al</i> ³	Unselected Israeli AJ prostate cancer cases.	87	2/87		1/87	No difference in carrier frequency between cases and controls. PSA and Gleason score at diagnosis higher among carrier patients than among non-carriers. Carrier cases were more likely to have multiple primary tumours
	Elderly Israeli AJ male controls	87	2/87	—	1/87	
Case series Lehrer <i>et al</i> ⁴	N. American AJ prostate cancer recruited from urology/radiation oncology clinics	60	0/60	—	0/60	No carriers identified
Nastiuk <i>et al</i> ⁵	N. American AJ with prostate cancer; pathology review.	83	1(1.2)	—	2 (2.4)	Carrier frequency not elevated compared with population estimates
Vazina <i>et al</i> ⁶	Unselected Israeli AJ men with prostate cancer; pathology review	95	2/87(2.3)	2/60(3.3)	1/86 (1.2)	No mutation detected in any patient diagnosed prior to the age of 55 years. While reported as a negative study, the results, when limited to the Ashkenazi subset, appear consistent with an increased risk among <i>BRCA1</i> mutation carriers compared with population estimates.

AJ, Ashkenazi Jew.

ovarian cancer among female founder mutation carriers. The incidence of prostate cancer in carriers in the WAS was elevated by the age of 50 years, was statistically significantly elevated by 67 years, and increased thereafter with age, approaching 30% by 80 years, suggesting both an overall excess in prostate cancer risk, and an increased risk of early onset disease among founder mutation carriers. While confidence limits were widely overlapping in the WAS analysis, the risk of prostate cancer appeared higher among individuals with *BRCA1* mutations than those with *BRCA2* mutations, and there was some suggestion of early onset disease among those with a *BRCA1* 5382insC mutation. In that study, the number of individuals with a family history of prostate cancer was 5/40 (12%), 5/20 (25%), 6/54 (11%), 16/114 (14%), and 364/4759 (8%) for 185delAG, 5382insC, 6174delT, any mutation, and non-carriers, respectively. Compared with non-carriers, those with a *BRCA1* 5382insC mutation and those with any founder mutation were at significantly increased risk of PrC ($p < 0.01$).

Warner and colleagues studied the risk of breast, ovarian and other cancers in the first degree relatives of 412 Ashkenazi Jewish women with breast cancer unselected for age or family history, who elected to undergo mutation testing.² Among first degree relatives of carriers of any founder mutation, the risk of prostate cancer was estimated to be 34% by the age of 85 years compared with 12.6% among non-carriers ($p = .05$). Interestingly, this study appeared to enrich for carriers of the *BRCA1* 185delAG mutation, despite the nearly equal frequencies of the *BRCA1* 185delAG and the *BRCA2* 6174delT mutations in the general Ashkenazi population. Only 15 (3.6%) of breast cancer

patients in this study carried a 6174delT mutation compared with 26 (6.3%) patients who carried a 185delAG mutation, perhaps reflecting variable penetrance of these two mutations for breast cancer. Differences in subject ascertainment could account for the observed differences in the estimated risk of prostate cancer in these two studies.

In our population based series, 3.2% of Israeli Ashkenazi men with an incident diagnosis of prostate cancer, unselected for family history of breast and/or ovarian cancer, carried an Ashkenazi founder mutation. No population controls were obtained as part of this study. This represents a major limitation of our study design. We have attempted to address this limitation through comparison with existing Ashkenazi groups for which age and sex specific estimates of the founder mutation rate are available, i.e., the WAS and the MECC.

The MECC data are derived from a population based study of Israeli men and, while relatively small, is the best available Israeli comparison group. There is no statistical difference in the mutation frequency among participants in the MECC (1.69%), which may over-represent Ashkenazi Jews immigrating from the former Soviet Union, and in that observed in the WAS (1.49%), composed predominantly of immigrants from Germany, Poland and Austria. Estimates of the Ashkenazi *BRCA* founder mutation prevalence have been reported for several populations. These rates are strikingly similar across Ashkenazi populations living in various geographical regions.^{1 7 8 14} We therefore thought it reasonable to combine the data from the MECC and WAS studies.

In the current study, we did observe an overall twofold risk of prostate cancer among founder mutation carriers.

While increased risk of prostate cancer among carriers of the *BRCA1* 185delAG and the *BRCA2* 6174delT mutations appeared to be comparable (within the constraints of small numbers), only the estimate for the all mutations combined achieved statistical significance ($p < 0.05$). No increased risk was associated with the 5382insC mutation in this study. The mean age at diagnosis among *BRCA1* mutation carriers did not differ significantly from that of the *BRCA2* mutation carriers, and neither differed significantly from the age at diagnosis among non-carriers. We found no evidence in this study to suggest an earlier than usual age of diagnosis in *BRCA* associated prostate cancer.

Interestingly, data from both the WAS and the MECC suggest that the *BRCA* founder mutation prevalence rate may decrease with age. This would suggest that employing the overall *BRCA* founder mutation frequency of approximately 2.4%^{7 8 14 1} as the comparison, rather than an age specific prevalence estimate, will result in an underestimation of the risk of prostate cancer. If one takes into account a possible decline in founder mutation prevalence with age, our findings are consistent with at most a 2–4 fold increase in the risk of prostate cancer among carriers of an Ashkenazi *BRCA* founder mutation.

Differences in the reported mutation specific risks probably represent statistical fluctuation related to small sample sizes. While a well designed, population based case–control study testing the hypotheses we explore here would be desirable, a study sufficiently powered to detect mutation specific risks is probably unfeasible. To detect reliably (with a power of 90%) an odds ratio of 1.5 for the separate mutations 185delA, 5382 insC, and 6174delT, one would need total sample sizes of approximately 25 000, 100 000 and 25 000 respectively, assuming equal numbers of cases and controls. However, there would be little clinical interest in such small odds ratios, as risks at this level would result in a very low population attributable risk (%), (calculated by $100 \cdot P \cdot OR / (P \cdot OR + 1 - P)$, where P = prevalence of mutation in the general population and OR = odds ratio) associated with *BRCA* founder mutation status. In our study, $P = 0.0156$ and $OR = 2.08$, so that the population attributable risk = 3.2%. Thus, although the relative risk of prostate cancer among carriers of *BRCA* Ashkenazi Jewish founder mutations is significantly elevated, founder mutations explain only a small percentage of all prostate cancers occurring among Ashkenazi men. In view of the moderately high incidence of prostate cancer among Ashkenazi Jews, the absolute number of cases attributable to the founder mutations is not inconsequential, but alternative aetiological explanations must be sought for the majority of prostate cancers.

This study represents the largest reported series of *BRCA* related prostate cancer to have undergone detailed, blinded histopathological review. Only one study to date has examined the clinical characteristics of prostate cancer diagnosed in Ashkenazi mutation carriers. In a small case-control study of Israeli Ashkenazi Jewish men with prostate cancer, Hubert and colleagues reviewed the pathology of three *BRCA* associated prostate cancers (two patients with *BRCA1* 185delAG mutations and one patient with a *BRCA2* 6174delT mutation). They suggested that these tumours appeared to be associated with biologically more aggressive disease. These cancers presented with higher PSA levels, higher Gleason scores and more advanced stage at diagnosis than did prostate cancers diagnosed in men without mutations.³

While data on initial stage and PSA at diagnosis were not available in our study, we found no evidence for distinctive clinical or pathological features among prostate cancer cases associated with an Ashkenazi founder mutation compared with prostate cancers occurring among non-carriers. It is

interesting to note that the majority of carriers were diagnosed by needle biopsy rather than at prostatectomy. We have insufficient information to address the basis for this association. We selected prostate cancer cases diagnosed from 1994–1995, a time period antedating the widespread use of PSA screening in Israel. Therefore, we believe it unlikely that a detection bias due to differential PSA screening among men with a family history of breast and/or ovarian cancer could account for this association. Some clustering of chronic prostatitis was noted among cases with mutations, which may suggest a predilection for *BRCA* associated prostate cancers to arise in this setting. We did not find a higher Gleason Score among mutation positive cases.

In summary, our findings are consistent with previous reports of a modest increase in the risk of prostate cancer associated with the Ashkenazi founder mutations. This risk is unlikely to exceed a fourfold increase. This level of risk may warrant consideration of male carriers of Ashkenazi founder mutations as candidates for inclusion in high risk prostate cancer screening and/or risk reduction studies. Our review of the pathological characteristics of founder mutation *BRCA* associated prostate cancer, while based on a relatively small number of cases, does not suggest that these cases display a more aggressive phenotype.

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ECHO

Genotype-phenotype analysis of the Crohn's disease susceptibility haplotype on chromosome 5q31

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Please visit the Journal of Medical Genetics website [www.jmedgenet.com] for a link to the full text of this article.

Background and aims: Recent molecular data suggest that genetic factors may underlie the disease heterogeneity observed in both ulcerative colitis (UC) and Crohn's disease (CD). A locus on chromosome 5q has been implicated in susceptibility to CD, and recently refined by linkage disequilibrium mapping to a conserved 250 kb haplotype (5q31). No data regarding the contribution of this locus to clinical phenotype exist. In this case control study, we investigated the contribution of this haplotype to both susceptibility and phenotype of CD and UC.

Patients and methods: We studied 330 Caucasian CD and 457 UC patients recruited from a single UK centre. Association with disease susceptibility and phenotype was analysed with haplotypes reconstructed from three single nucleotide polymorphisms chosen to span this susceptibility region. Evidence for possible genetic epistasis between *IBD5* and *NOD2/CARD15* was sought.

Results: Linkage disequilibrium across this region was confirmed, with two haplotypes comprising 88% of all chromosomes. Susceptibility to CD, but not to UC, was associated with homozygosity for a common haplotype, H2 ($p_c = 0.002$; relative risk (RR) 2.0). Genotype-phenotype analyses demonstrated that this association was particularly strong in patients with perianal disease ($p_c = 0.0005$; RR 1.7), especially in individuals homozygous for this haplotype ($p_c = 0.0005$; RR 3.0). Importantly, no association with H2 was found in 186 patients without perianal disease. No evidence of epistasis between *IBD5* and *NOD2/CARD15* was demonstrated.

Conclusions: The *IBD5* risk haplotype is associated with CD only. Genotype-phenotype analysis reveals that the strongest association is observed in patients with perianal CD. While the precise gene involved is unclear, these data provide further molecular evidence for a genetic basis of the clinical heterogeneity of CD.

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LETTER TO JMG

Congo red, doxycycline, and HSP70 overexpression reduce aggregate formation and cell death in cell models of oculopharyngeal muscular dystrophy

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The formation of intracellular amyloid-like inclusions by mutant proteins is a feature of two groups of codon reiteration diseases, for which there are currently no treatments. The first group that was described includes the nine known neurodegenerative conditions caused by polyglutamine (polyQ) repeat expansions resulting from CAG trinucleotide repeat mutations, exemplified by Huntington's disease (HD).¹ HD is caused by a tract of more than 37 uninterrupted polyglutamines in exon 1 of the HD gene product, huntingtin. Genetic and transgenic studies are consistent with a model where expanded polyglutamines cause disease by conferring a novel toxic function on the disease proteins.^{1–3}

The second type of codon reiteration mutation results in autosomal dominant oculopharyngeal muscular dystrophy (OPMD).⁴ OPMD is caused by the abnormal expansion of a (GCG)_n trinucleotide repeat in the coding region of the polyadenine binding protein 2 gene (*PABP2*): a (GCG)₆ repeat is expanded to (GCG)_{8–13} in most patients. In some rare cases, insertion mutations such as (GCG)₆GCA(GCG)₂, (GCG)₆GCA(GCG)₃ and (GCG)₆(GCA)₃(GCG)₂ are seen.^{5,6} In *PABP2*, (GCG)₆ codes for the first six alanines in a homopolymeric stretch of 10 alanines. Thus, disease is associated with expansions of 12 or more uninterrupted alanines in this nuclear protein. OPMD is characterised by aggregates in muscle cell nuclei comprising mutant *PABP2* as a major component.^{4,7–9}

The role of inclusions in these diseases has been vigorously disputed.¹ Nevertheless, strategies that target protein misfolding frequently reduce aggregate formation and cell death in parallel. In mammalian cell based models of both polyglutamine and polyalanine diseases, the mutant proteins are much more prone to aggregate formation than their wild-type counterparts and cause significantly more cell death.^{10,11} In such models, aggregate formation and cell death can be reduced by overexpressing yeast and bacteria derived chaperones that do not appear to protect against some other cell death pathways.^{11–13} A causal role for aggregation in cell death in tissue culture models of OPMD is supported by complementary data from our lab and Rouleau's group.^{11,14} Rouleau and colleagues found that oligomerisation of *PABP2* is mediated by two potential oligomerisation domains (ODs)—deletions in either of these domains inactivated oligomerisation of mutant *PABP2* and also reduced the cell death caused by this protein.¹⁴

The similarities between polyglutamine diseases and OPMD have led us to explore whether strategies that protect against polyglutamine aggregation or toxicity are also effective in OPMD models. Our previous studies suggested that mammalian heat shock proteins might be able to play similar roles in both diseases.^{11,15} Members of the HSP70 and HSP40 family members are recruited to polyQ inclusions in vivo and in cell models.^{11,15–21} We have tested

Key points

- Intracellular amyloid-like inclusions formed by mutant proteins result from polyglutamine expansions in Huntington's disease (HD) and polyalanine expansions in polyadenine binding protein 2 (*PABP2*) in oculopharyngeal muscular dystrophy (OPMD).
- Here we show further parallels between these diseases and suggest therapeutic strategies for OPMD. Like polyglutamine diseases, HSP70 and HDJ-1 colocalised with *PABP2* aggregates in OPMD patient muscle tissue and overexpression of HSP70 reduced mutant *PABP2* aggregate formation. Aggregate formation and cytotoxicity in cell models of OPMD were reduced by Congo red or doxycycline.
- Our data highlight the therapeutic potential of these compounds in oculopharyngeal muscular dystrophy.

if this is the case in OPMD patients, as we had previously shown that this occurred in a cell model of OPMD.¹¹ We have previously shown that HDJ1, an HSP40 family member, reduced aggregate formation and cell death in cell models of HD and OPMD.^{11,15} Since HDJ1 is a co-chaperone for HSP70, we have now tested if HSP70 chaperone is effective in cell models of OPMD, as it can be effective in HD.¹⁵

Since it may be possible to treat these diseases with compounds that reduce aggregate formation, we have been testing a number of anti-amyloid compounds in cell based models of OPMD (where no animal models have been published). We tested Congo red, as data from Ross and colleagues suggested that it blocked the conversion of mutant huntingtin protofibrils into mature fibrils²² and Sanchez et al showed that Congo red reduced aggregation and cell death in HD cell models.²³ The latter study also reported that infusion of Congo red into an HD mouse model by the intraperitoneal and intracerebroventricular routes improved survival, weight loss, and motor function, compared with untreated mutant mice.²³ These data provided important insights into the role of inclusions polyglutamine disease pathology and suggested that the beneficial effects of Congo red were due to its

Abbreviations: DAPI, 4', 6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; Dox, doxycycline hydrochloride; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; HD, Huntington's disease; HSP, heat shock protein; OD, oligomerisation domain; OPMD, oculopharyngeal muscular dystrophy; *PABP2*, polyadenine binding protein 2; polyQ, polyglutamine

anti-aggregate properties. Unfortunately, the therapeutic potential for Congo red in HD may be minimal, because of its very poor blood brain barrier permeability.²⁴ On the other hand, brain penetrance would not be an issue for a muscle disease like OPMD. We also tested doxycycline as an anti-aggregate compound, since previous studies had suggested that tetracyclines were anti-amyloidogenic.²⁵

MATERIALS AND METHODS

Cell culture and transfection

African green monkey kidney (COS-7) cells were grown in Dulbecco's modified Eagle's medium (DMEM 5471) (Sigma) supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, and 2 mM L-glutamine at 37°C, 5% CO₂. For transfection of plasmid DNA, cells were seeded on coverslips at 0.6–1×10⁵ per well, in 6-well plates, the day before transfection. Cells were transfected with Lipofectamine (Life Technologies) according to the manufacturer's instructions. Constructs are described in refs 11 (PABP2) and 15 (HSP70).

Compounds and treatment

Congo red (Sigma) was dissolved in DMEM 5671 (Sigma) with a stock concentration of 10 mg/ml. Doxycycline hydrochloride (Dox) (Sigma) was dissolved in water with a stock concentration of 100 mg/ml. All the stock solutions were sterilised through a filter with a 0.2 µm pore diameter. The compounds were diluted to the required concentration in the cell culture medium immediately before use. At 24 h after transfection, the compounds were added to the cells at different concentrations. The cells were incubated with the compounds for another 24 h and then washed with 1× phosphate buffered saline, fixed in 4% paraformaldehyde for 20 min, mounted in antifade reagent supplemented with 3 µg/ml of 4', 6-diamidino-2-phenylindole (DAPI) to allow visualisation of nuclear morphology.

Western blotting analysis

Cells were lysed with Laemmli buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 5% β-Mercaptoethanol, 10% Glycerol, 0.01% Bromophenol Blue). Lysates were subjected to SDS-polyacrylamide gel (10%) electrophoresis and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, UK). The membrane was incubated with mouse polyclonal anti-HSP70 (StressGene, 1:1000), mouse polyclonal anti-HDJ-1 (StressGene, 1:1000) and rabbit anti-actin (Sigma, 1:2000), respectively. Blots were then incubated with peroxidase conjugated antimouse or antirabbit IgG antibody (Amersham Pharmacia Biotech, 1:1000). The proteins were detected using the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech). Protein loading was corrected by reprobing blots for actin.

Immunohistochemistry

Studies on human muscle samples were performed with the approval of ethics committees in Cambridge and Kumamoto. We have analysed frozen tissue sections from a muscle biopsy of the right sternohyoideus from an OPMD patient (female, 62 years old, with a 15 polyalanine expansion mutation in the PABP2 gene (genotype: (GCG)₁₁/(GCG)₆), the right gastrocnemius muscle from a patient (female, 61 years old, 15 polyalanines in PABP2; (genotype: (GCG)₁₁/(GCG)₆), the right deltoid muscle from a normal control (20 years old) and the right biceps muscle from a patient with dentatorubral pallidolusian atrophy (non-OPMD disease control, male, 62 years old). The sections were fixed in 4% paraformaldehyde fixative for 10 min and then blocked with 5% horse serum for 1h. The colocalisation of endogenous PABP2 with HSP70, or PABP2 with HSP40 was detected by using the rabbit

polyclonal anti-PABP2 antibody²⁶ (1:200, kindly provided by Professor Elmar Wahle, Halle, Germany) together with the monoclonal mouse anti-HSP70 (1:200), or by using anti-PABP2 antibody together with the monoclonal mouse anti-HDJ-1 (1:200). The sections were then incubated with the mixture of antirabbit Texas red conjugated goat IgG antibody (Molecular probes, USA) and antimouse FITC conjugated goat IgG antibody (Alexa Fluor G488, Molecular probes, USA).

Confocal microscopy

Fluorescently labelled samples were analysed using the laser scanning microscope Zeiss LSM410 equipped with an argon ion laser (wavelength, 488 nm) to excite FITC fluorescence, and a helium neon laser (wavelength, 543 nm) to excite Texas red fluorescence. For double labelling experiments, images from the same focal plane were sequentially recorded in different channels and merged to confirm colocalisation.

Measurement of aggregate formation and abnormal cell nuclei

Cells were fixed with 4% paraformaldehyde at 72 h after transfection and counterstained with DAPI. Aggregate formation and nuclear morphology were assessed with a fluorescence microscope. Two hundred enhanced green fluorescent protein (EGFP) expressing cells were counted (with the observer blinded to the slide identity) across the centre region of the slides, to quantify all types of polyalanine aggregates in the cells. We assessed the proportions of PABP2-A17 expressing cells that contained one or more inclusions. We considered cells to have inclusions if the green fluorescent protein (GFP) was abnormally concentrated and differed from the nuclear speckled appearance typically seen with the normal PABP2-A10. Cells were considered dead if the DAPI-stained nuclei showed apoptotic morphology (fragmentation or pyknosis). Pyknotic nuclei are typically <50% diameter of normal nuclei and show obvious increased DAPI intensity. We have demonstrated that these criteria are specific for cell death, as they show a very high correlation with propidium iodide staining in live cells.¹⁵

Statistical analysis

As we and others have described previously,^{10–13} pooled estimates for the changes in inclusion formation resulting from perturbations assessed in multiple experiments were calculated as odds ratios with 95% confidence intervals:

$$\left(\frac{\% \text{ cells expressing construct with inclusions in perturbation conditions}}{\% \text{ cells expressing construct without inclusions in perturbation conditions}} \right) \div \left(\frac{\% \text{ cells expressing construct with inclusions in control conditions}}{\% \text{ cells expressing construct without inclusions in control conditions}} \right)$$

Odds ratios and p values were determined by unconditional logistical regression analysis using the general log/linear analysis option of SPSS ver 6.1 software (SPSS, Chicago, USA). p<0.05 was considered to be statistically significant.

RESULTS

To test if the intranuclear inclusions in OPMD patients sequester heat shock proteins (HSPs) in a manner similar to what we described in cell models, we used double staining

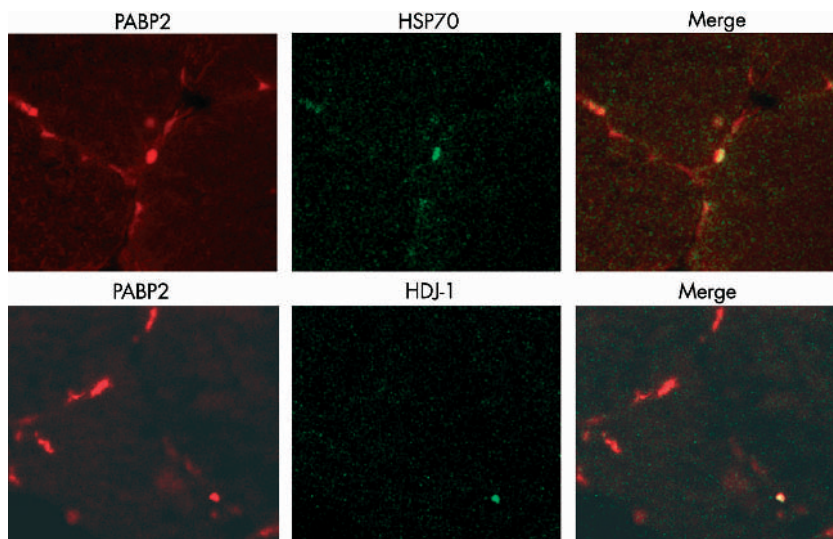


Figure 1 Colocalisation of HSP70 and HDJ-1 with nuclear PABP2 inclusions in muscle biopsies from OPMD patients. Frozen sections of muscle biopsies from OPMD patients were processed for immunohistochemistry. Texas red conjugated (red) second antibody was used to label PABP2 and FITC conjugated (green) secondary antibody was used to label HSP70 and HDJ-1. Colocalisation was confirmed by analysing images from the same focal plane sequentially recorded in different channels by confocal microscopy.

immunohistochemistry to allow simultaneous analysis of both mutant PABP2 and the relevant HSPs. Fig 1 shows colocalisation of HSP70 and HDJ-1 (HSP40 family) in inclusions in the DAPI stained nuclei of OPMD muscle. Similar findings were observed in samples from two OPMD patients. No colocalisation of these HSPs with PABP2 was seen in two control muscle samples (data not shown). The anti-PABP2 antibody we have used²⁶ has been previously used to distinguish between cells with and without PABP2 aggregates.^{9, 14} The strong nuclear staining for the chaperones correlated strongly with the cells with nuclear aggregates (fig 1).

Previously, we showed that GFP tagged mutant PABP2 with 17 alanines (PABP2-A17) resulted in increased intranuclear aggregate formation and cell death, compared with otherwise identical constructs with 10 alanines¹¹. HSP70 co-expression with GFP tagged mutant PABP2 with 17 alanines (PABP2-A17) significantly reduced the proportion of GFP positive cells with aggregates, compared with cells cotransfected with an empty vector control (pFlag-empty) (fig 2). HSP70 overexpression reduced the toxicity of mutant PABP2 in parallel with the reduction in

aggregates (fig 2). Fig 2B shows that total HSP70 levels increase in cells transfected with HSP70, or in cells treated with sodium arsenite, which induces a heat shock response (as a positive control).

We considered Congo red and doxycycline as chemical chaperones with the potential to reduce mutant PABP2 aggregation and toxicity. COS-7 cells (the only cell model that has been reported to show aggregation and cell death as a readout for mutant OPMD toxicity) were transfected with GFP-tagged mutant PABP2 with 17 alanines (PABP2-A17). Twenty four hours after transfection, we added different concentrations of Congo red and doxycycline for the next 24 h and then assessed the proportions of GFP positive cells with aggregates and cell death (as scored by apoptotic nuclear morphology). We found that both Congo red and doxycycline reduced aggregation and cell death caused by mutant PABP2 (fig 3A). These effects were not caused by these compounds inducing raised levels of stress inducible HSPs, like HSP70 (fig 3B). While Congo red did not protect against cell death caused by incubating cells in staurosporine (2.5 μ M) or H₂O₂ (400 μ M)¹¹, protective effects were observed with doxycycline (fig 3C). In these experiments,

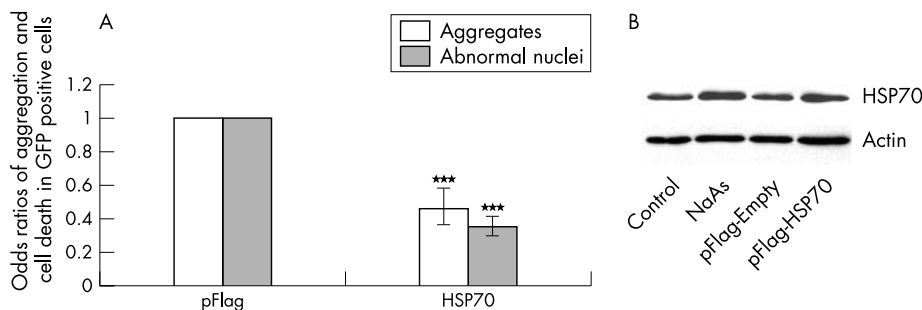


Figure 2 HSP70 overexpression suppresses aggregation of PABP2-A17 and its cytotoxicity. (A), PABP2-A17 (0.5 μ g) was cotransfected with an expression vector for HSP70 (pFlag-HSP70, 1.5 μ g) or an empty vector (pFlag-empty, 1.5 μ g). Cells were fixed and stained with DAPI 72 h after transfection. Aggregate formation and nuclear fragmentation/condensation were counted. The odds ratios were derived from 2–4 independent experiments, each done in triplicate. The error bars represent the 95% confidence intervals for the odds ratios. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$; not significant, $p > 0.05$. (B), Western blot showing expression of HSP70 in transfected COS-7 cells. COS-7 cells were either transfected with 2 μ g PABP2-A17, treated or non-treated with sodium arsenate (NaAsO₂, 50 μ M, treated for 1 h) or cotransfected with PABP2-A17 (0.5 μ g) and pFlag-HSP70 (1.5 μ g), PABP2-A17 and pFlag-empty (1.5 μ g). NaAsO₂ was used as a positive control to stimulate HSP70 expression.

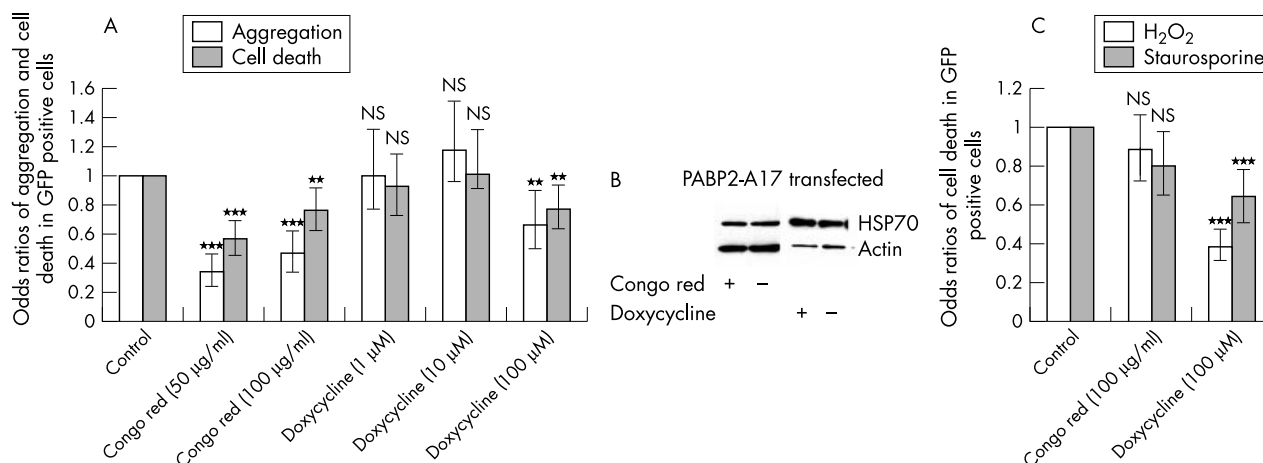


Figure 3 Congo red and doxycycline (Dox) reduce aggregation and cell death in cell models of OPMD and HD. (A) COS-7 cells were transfected with PABP2-A17. Congo red or Dox with different doses were added 24 h after transfection. Cells were incubated with the compounds for another 24 h and then fixed and stained with DAPI. The proportions of cells with aggregates and nuclear fragmentation or condensation were determined. The odds ratios were derived from 2–4 independent experiments, each done in triplicate. The error bars represent the 95% confidence intervals for the odds ratios. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$; not significant, $p > 0.05$. The 95% confidence intervals for 50 µg/ml and 100 µg/ml Congo red overlap, suggesting that there are no significant differences between these doses. (B) Congo red and Dox do not stimulate the expression of HSP70. COS-7 cells were transfected with PABP2-A17. 100 µg/ml of Congo red or 100 µM of Dox was added to the cells 24 h after transfection. Cells were harvested after another 24 h, Western blotting was carried out, and the expression level of HSP70 was detected. (C) Effects of Congo red (100 µg/ml) and Dox (100 µM) on cell death caused by pro-apoptotic agents. COS-7 cells were transfected with the vector pEGFP-C1 and the Congo red and doxycycline were added 24 h after transfection. After another 24 h, either H₂O₂ or Staurosporine were added to the cells (for 4 h and 6 h, respectively) before fixing the cells. Nuclear fragmentation and condensation were determined in GFP positive cells. The odds ratios were derived from 2–4 independent experiments, each done in triplicate. The error bars represent the 95% confidence intervals for the odds ratios. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$; not significant, $p > 0.05$.

cells were treated in the same protocol as described previously¹¹, and staurosporine and H₂O₂ were added in the final 6 h and 4 h, respectively, of the experiment.

Discussion

Both polyglutamine diseases like HD and polyalanine expansion mutations in PABP2 are associated with intracellular inclusions, whose appearance correlates with cell death in model systems. In this study we have extended the parallels between these different types of codon reiteration mutations by demonstrating HSP70 and HDJ-1 colocalisation in PABP2 inclusions *in vivo*, which has been previously observed in HD and related polyglutamine diseases^{15–21}. The failure to detect colocalisation of these HSPs with PABP2 in wild-type samples or outside the nucleus in OPMD tissues is likely to be because the HSP preferentially associate with the aggregated protein and because PABP2 is predominantly nuclear in steady state. HSP70 overexpression resulted in a reduction of mutant PABP2 and HD exon 1¹⁵ aggregation and cytotoxicity. While this paper was being written, Abu-Baker et al published data also showing colocalisation of HSP70 with OPMD aggregates and reduction of these aggregates with overexpression of this chaperone.²⁷ Since HSP70 can directly protect against certain apoptotic pathways, for instance by inhibiting cytochrome c-mediated caspase activation,^{28–29} it is important to be cautious about inferring a causal relationship between reduced aggregation and cell death in this context. Indeed, this cell death pathway is activated and contributes to the cytotoxicity in HD cell models.¹⁵

The reduction in aggregation and cell death mediated by Congo red and doxycycline against mutant PABP2 suggests that these compounds may have therapeutic potential, especially for OPMD. The concentrations that we have used are compatible with previous studies in HD models. We obtained significant reductions in aggregation and cell death caused by mutant PABP2 with 50 µg/ml (70 µM) Congo red, while Sanchez and colleagues reported reduction in their HD cell models with 100µM.²³ Recently, Smith et al reported that

doxycycline reduced aggregation in a slice culture model of HD at 30µM,³⁰ while we observed protection in our OPMD model with 100µM. Doxycycline appears to be protective by reducing aggregation and also by reducing susceptibility to cell death pathways induced by apoptotic inducers like staurosporine and H₂O₂, the latter effect compatible with the data of Chen et al who showed minocycline reduced caspase activation in a mouse model of HD.³¹ However, since tetracyclines are predicted to protect against apoptosis by inhibiting caspases and caspase 1 is interleukin-1 β -converting enzyme, the effects of tetracyclines may also be due to anti-inflammatory activity in certain *in vivo* disease models. While Congo red may have limited utility in HD due to its poor blood brain barrier permeability,²⁴ it may be useful for diseases associated with aggregation outside the central nervous system, such as OPMD. Congo red appears to be a general amyloid protein ligand.³¹ In the case of HD, it blocks the conversion of protofibril to mature fibre formation in the aggregation process.²² Modelling studies reveal that Congo red is likely to break the continuity of the ordered structures of the β -sheets characteristic of polyalanine expansions,^{32–33} thus providing a molecular explanation for the effect we observed with the mutant PABP2 protein. The parallel protection that Congo red affords against the cytotoxicity of expanded polyglutamines and polyalanines is consistent with the idea that abnormal protein aggregation and accumulation may be deleterious in all intracellular amyloidoses, irrespective of the primary mutation.

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CORRECTION

Giusti RM, Rutter JL, Duray PH, *et al.* *J Med Genet* 2003;**40**:787–792. During the production process errors were introduced into Table 1 of this paper. The odds ratio value for the *BRCA1* 5382insC mutation in the WAS + MECC studies should read 0.95. The 95% CI for the *BRCA2* 6174delT and the total positive frequency should read 0.89–4.56 and 1.18–3.65, respectively.