Letters to the Editor

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Modeling the Probability That Ashkenazi Jewish Women Carry a Founder Mutation in BRCA1 or BRCA2

To the Editor:

The Washington study (see Struewing et al. 1997) currently provides the closest to population-based data on the prevalence of the three common BRCA1 (MIM 113705) and BRCA2 (MIM 600185) founder mutations in Ashkenazi Jewish women, so the most use must be made of it. In this respect, Hartge et al. (1999) should be congratulated for presenting raw data in their recent publication in the *Journal*.

Hartge et al. (1999) use the classification-and-regression tree (CART) approach to identify the "most important" predictors of mutation status. They conclude that a previous diagnosis of breast or ovarian cancer was the most important discriminator. For women with such a cancer history (i.e., affected individuals), the next most important predictor was apparently age at diagnosis, after which "family history discriminated relatively little" (p. 965). In contrast, for men and women without a personal history of breast or ovarian cancer (i.e., unaffected individuals), family history "best discriminated" between carriers and noncarriers. In their table 2, Hartge et al. present the most important subcategories of risk, derived by CART, along with the observed frequency in each cell. The number of carriers in some cells is small, however, making some inferences imprecise.

The CART method for ranking predictors in terms of "importance" is influenced by the distribution of predictive factors, not just by the size of the effect itself. It is of interest, therefore, to estimate the *strengths* of putative predictive factors with regard to the probability of being a carrier, both for affected and for unaffected individuals. One way is by the use of multiple logistic regression, in which the effects are estimated on the log-odds scale. Not only does this statistical approach give explicit estimates of the magnitude of the effects of predictors, along with measures of the precision of these estimates (such as standard errors [SEs] and confidence intervals [CIs]), it also allows one to summarize the data in terms of as few parameters as is possible for extraction of the most information. Such a "parsimonious" model uses all the available information to produce a general picture of the strength and nature of the major predictive factors. (Hartge et al. [1999] do not report the findings of logistic-regression analyses, apparently because they found that the estimated frequencies were "substantially higher" than the observed frequencies in groups with observed prevalence >20%. As can be seen from their tables 3 and 4, however, this only applied to 34 cases at age <40 years at diagnosis, 20 of age 40–49 years at diagnosis, and 10 of age 50–59 years at diagnosis, i.e., <2% of the 3,742 women in the total data set.)

Tables 1 and 2 present the observed prevalence of mutation carriers, extracted from tables 3 and 4 of Hartge et al. (1999), both for women with and for women without a diagnosis of *breast* cancer, by age at diagnosis or age at testing. It also categorizes women according to their family history of *breast* or *ovarian* cancer, defined either by the number of first-degree relatives with breast cancer or by whether any first-degree relative had breast cancer diagnosed at age <50 years (referred to there as "early diagnosis"). Several points are readily seen:

1. The prevalence increases for each affected first-degree relative, for both affected *and* unaffected individuals; for example, as the number of affected relatives increases from 0 to 1 to 2, the prevalence in affected individuals increases from 1 in 14 to 1 in 8 to 1 in 4; in unaffected individuals, it increases from 1 in 80 to 1 in 28 to 1 in 16.

2. In women with a family history, the prevalence is greater if at least one of the affected relatives had an early diagnosis, for both affected and unaffected individuals.

3. There is a substantial difference, in prevalence between affected and unaffected individuals; this applies to each of the family-history and age categories, although it is not necessarily of the same strength in each category.

4. There is evidence for an "age" effect in both affected *and* unaffected individuals; for example, in women with a family history of breast cancer, the prevalence decreases from 43% to 16% to 8% as age *at diagnosis* increases from age <40 to 40–49 to ⩾50 years. In unaffected individuals, it decreases from 8% to 4%

Table 1

Prevalence of the Three BRCA1 and BRCA2 Founder Mutations in Ashkenazi Jewish Women Who Have Never Had a Diagnosis of Breast or Ovarian Cancer, as a Function of Family History of Breast Cancer and Age at Testing (Hartge et al. [1999], table 3)

	Proportion (%) of Individuals with BRCA1/BRCA2 Founder Mutations When				
	No. of First-Degree Relatives with Breast Cancer Is			Early Diagnosis in Affected Relatives Is	
Age at Testing (years)	0	1	⩾2	Not Present	Present
<40	9/566 (1.6)	10/123 (8.1)	0/1 (0)	5/62 (8.1)	5/62 (8.1)
40–49	14/888 (1.6)	9/217 (4.1)	0/7 (0)	3/143 (2.1)	6/81 (7.4)
50–59	8/636 (1.3)	4/163 (2.5)	2/12 (17)	4/119 (3.4)	2/56 (3.6)
⩾60	4/615 (.7)	1/163 (.6)	1/28 (3.6)	1/124 (.8)	1/67 (1.5)
Total	35/2,705 (1.3)	24/666 (3.6)	3/48 (6.3)	13/448 (2.9)	14/266 (5.3)

to 2% as the age *at testing* increases from age ⩽40 to 40–49 to ⩾50 years.

Multiple logistic-regression analyses were conducted for the data in tables 1 and 2, by use of the software GLIM (Baker and Nelder 1978). Likelihood-ratio tests were used to test between nested models, and the scaled deviance was used as a goodness-of-fit test (McCullagh and Nelder 1983).

First, family history was analyzed in terms of the number of affected first-degree relatives. (Note that there were no women of age <40 years in the data set who both were affected and had more than one affected first-degree relative, so this category has been deleted from the analysis.) In neither affected nor unaffected individuals was there evidence for an interaction between the effects of family history and "age" ($\chi^2_5 = 3.3$, $P = .6$; and $\chi^2_6 = 6.2$, $P = .4$, respectively). Furthermore, the effects of both age and family history were well represented by one parameter each: a linear effect on the log-odds scale per each age category (both $\chi^2_2 < 2$, $P < .4$) and per each affected relative (both $\chi^2_1 < 0.1$, $P < .8$). Within each group, there was no evidence that a logistic model that involved such linear effects for age and number of affected relatives gave an unacceptable fit ($\chi^2_9 = 7.5$, $P = .6$; $\chi^2_8 = 5.3$, $P = .7$, respectively). On the log-odds scale, the effect of age at diagnosis in affected individuals was −0.970 (SE, 0.257) per age category ($P < .001$), and the effect of age at testing in unaffected individuals was −0.434 (0.130) per age category ($P < .001$). The effect per affected relative was 0.826 (0.327) in affected individuals ($P = .01$) and 1.061 (0.222) in unaffected individuals ($P < .001$); that is, this family history effect was no different between affected and unaffected individuals ($P = .6$).

When data from affected and unaffected individuals were combined, the parsimonious model gave an acceptable fit ($\chi^2_{18} = 13.2$), and the linear predictor (with SEs of regression parameters in parentheses) was

$$\begin{aligned} &0.986\,(0.185) \times \text{no. of affected relatives} + \\ &[-4.319\,(0.380) - 0.426\,(0.129) \\ &\quad \times \text{age at testing in unaffecteds}], \text{ or} \\ &[-1.432\,(0.606) - 0.993\,(0.257) \\ &\quad \times \text{age at diagnosis in affecteds}] \,. \end{aligned} \tag{1}$$

(Here age is categorized as 1–4, respectively, for the groups age <40, 40–49, 50–59, and ⩾60 years.) The effect of having one affected relative was 0.983 (0.232), no different from that of having a second, which was 0.956 (0.503); $P > .9$. The average effect per affected relative was equivalent to the odds of being a mutation carrier increasing by $\exp(0.986) = 2.7$-fold (95% CI, 1.9–3.9) for each affected first-degree relative, irrespective of whether the woman was affected. In affected in-

Table 2

Prevalence of the Three BRCA1 and BRCA2 Founder Mutations in Ashkenazi Jewish Women Who Have Had a Diagnosis of Breast Cancer, as a Function of Family History of Breast Cancer (in a First-Degree Relative) and Age at Diagnosis (Hartge et al. [1999], table 4)

	Proportion (%) of Individuals with BRCA1/BRCA2 Founder Mutations When				
	No. of First-Degree Relatives with Breast Cancer Is			Early Diagnosis in Affected Relatives Is	
Age at diagnosis (years)	0	1	⩾2	Not Present	Present
<40	6/27 (22)	3/7 (43)	...	1/3 (33)	2/4 (50)
40–49	6/77 (8)	3/25 (12)	2/7 (29)	2/23 (9)	3/9 (33)
50–59	2/59 (3)	3/21 (14)	1/2 (50)	2/15 (13)	2/8 (25)
⩾60	1/47 (2)	0/22 (0)	0/3 (0)	0/15 (0)	0/10 (0)
Total	15/210 (7)	9/75 (12)	3/12 (25)	5/56 (9)	7/31 (23)

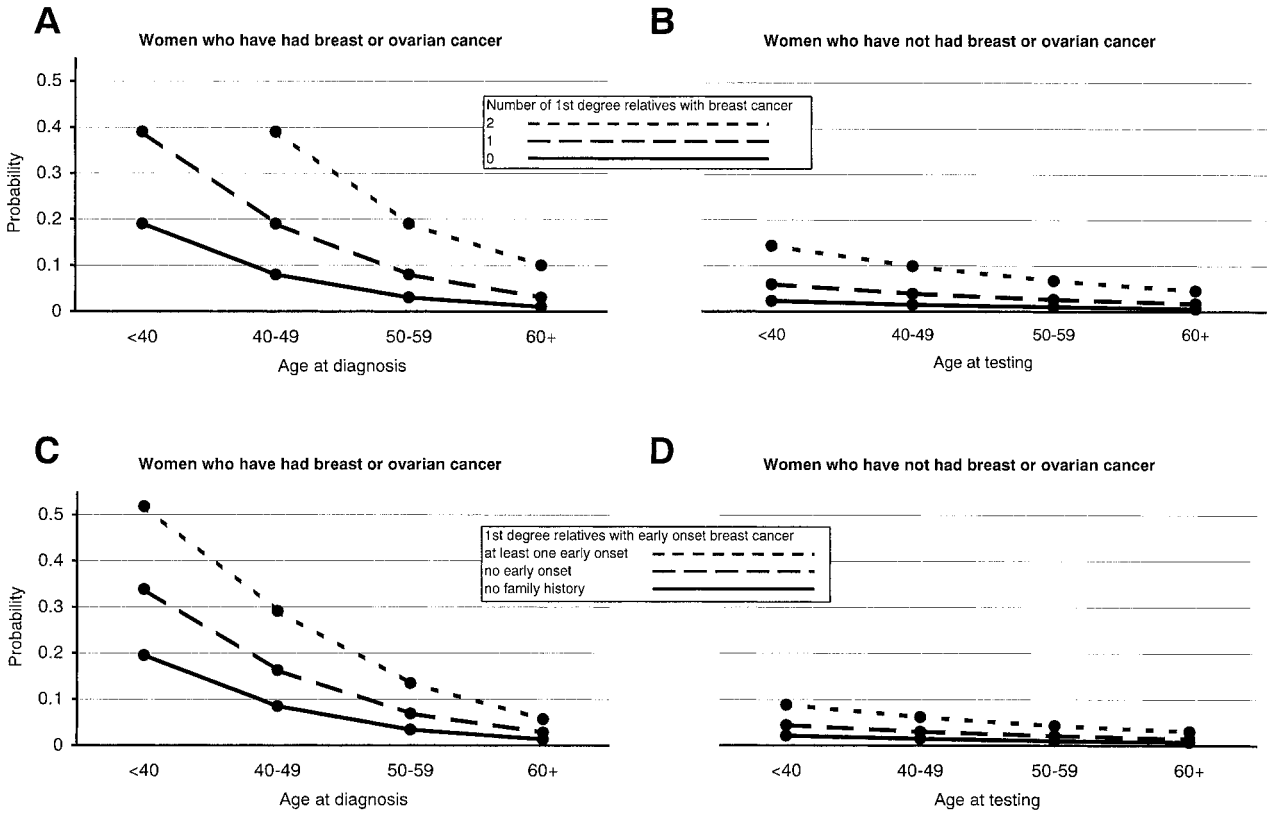

Figure 1 Estimated probability of carrying a founder mutation in BRCA1 or BRCA2 for Ashkenazi women. *A* and *C,* women who have had breast or ovarian cancer, as a function of age at diagnosis. *B* and *D,* women who have not had breast or ovarian cancer, as a function of age at testing. In *A* and *B,* family history is defined in terms of number of first-degree relatives with breast cancer; in *C* and *D,* family history is defined in terms of whether any first-degree relative had breast cancer at age <50 years (early diagnosis). (Note that because, in the data set, there were no women age <40 years who were both affected and who had more than one affected first-degree relative, a probability has not been assigned to that category.)

dividuals, it decreased by 0.4-fold (95% CI, 0.2–0.6) per category of age at diagnosis, whereas in unaffected individuals, it decreased by 0.7-fold (95% CI, 0.5–0.8) per category of age at testing.

By a simple reparameterization, it can be shown that the odds were greater in affected than in unaffected individuals with the same number of affected relatives, but the difference depended on age at diagnosis/testing, decreasing from ~10-fold (95% CI, 3.7–28), for women of age <40 years, to 1.9-fold (95% CI, 1.4–2.4), for women of age ⩾60. The CIs indicate that the effect of being affected was clearly significant at all ages.

Second, the same analysis was carried out again, this time with women with a family history categorized as 0, for no relatives affected; 2, for having at least one relative with "early diagnosis"; and 1 otherwise. As before, in neither affected nor unaffected individuals was there evidence for an interaction between the effects of family history of breast cancer and age ($\chi^2_6 = 3.3$, $P = .6$; $\chi^2_6 = 4.0$, $P = .7$, respectively). Furthermore, the effect of family history was well represented by a linear effect on the log-odds scale, per each family-history category (both $\chi^2_2 < 0.4$, $P = .8$), that was no different between affected and unaffected individuals; 0.752 (0.272) versus 0.741 (0.155), $P > .9$.

Combining affected and unaffected individuals, the parsimonious model again gave an acceptable fit ($\chi^2_{19} = 10.7$, $P = .9$), and the linear predictor was

$$0.744\ (0.135) \times \text{family history category} + [-4.184(0.371) - 0.383(0.128) \times \text{age at testing in unaffected individuals}], \text{ or } [-1.199(0.582) - 0.862(0.251) \times \text{age at diagnosis in affected individuals}]; \quad (2)$$

that is, the estimated effects were very similar to those in equation (1). (The effect of family history was 0.775 [0.287] in going from no family history to category 1, no different from the effect of 0.705 [0.341] in going from category 1 to category 2; $P > .8$.)

For both equation (1) and equation (2), there was no evidence for a poor fit, in that the estimated probabilities were not unduly disparate from the observed prevalences, in any category. In particular, for the two categories with the highest estimated probabilities according to equation (1), .39 in both cases, the observed values were .43 and .29 from samples of $n = 7$ each. For equation (2), the three highest estimated probabilities were .52, .34, and .29, similar to the observed prevalences of .50, .33, and .33 from samples of $n = 4$, 3, and 9, respectively.

The estimated probability of being a carrier, derived from the parsimonious models (1) and (2) above, are plotted, for the various categorizations in figure 1*A–D*. It can be seen that, for women with the same family history, the estimated probability in affected versus unaffected individuals was approximately four times greater for women in the youngest age category, but was only approximately two times greater for those in the oldest age category. That is, the effect of being affected depended on "age," a feature not discussed by Hartge et al. (1999).

From figure 1*A* and *B*, it can be seen that the probability approximately doubled for each affected first-degree relative. Figure 1*C* and *D* shows that, given a family history, the probability approximately doubled again if any of the affected relatives had an early diagnosis. These effects of family history were evident—and of the same magnitude on the log-odds scale—in both affected and unaffected individuals, another point not brought out by the CART analysis. We cannot, however, on the basis of the published data, test whether these effects were independent of one another.

In affected individuals, the probability approximately halved for each decade of age at *diagnosis*, whereas in unaffected individuals it approximately halved for every 2 decades of age at *testing*. The latter effect was not apparent from the CART analysis.

Thus, our analyses of the published Washington data suggest simple rules for how the probability that an Ashkenazi Jewish woman has inherited a founder mutation in BRCA1 or BRCA2 depends on her personal or family history of breast cancer. The effect of a having a single first-degree relative with breast cancer is generally a modest increase in prevalence equivalent to an odds ratio of either 2.7 from equation (1) or 2.2 from equation (2), both of which are similar to our estimate of 2.6 for a defined set of protein-truncating mutations in BRCA1 and BRCA2 that cause breast cancer in Australian women at age <40 years (Hopper et al. 1999). It would be interesting to do a similar analysis for a family history of ovarian cancer; table 1 of Hartge et al. (1999) suggests that having at least one first-degree relative with ovarian cancer is associated with an odds ratio of 4.1 (95% CI, 2.1–8.1) for having a founder mutation. Our analysis above has shown that this effect was no different from that of having a first-degree relative with breast cancer ($P = .3$).

Hartge et al. (1999) note that their observed probabilities for a given number of affected relatives were, in general, *less* than those derived from data collected in the setting of a cancer family clinic (Couch et al. 1997; Shattuck-Eidens et al. 1997). The discrepancy is likely to be due to the fact that the typical multiple-case families that come to such clinics also have cases with early-onset disease and other features—such as multiple primary cancers or breast and ovarian cancer in the same individual—that are also likely to increase their probability of being a carrier, as suggested by table 2 of Hartge et al. (1999). Without knowledge of *all* these predictors of mutation status it is not possible to make a valid inference from the clinic setting to the population.

The majority of "hereditary" cases of breast and ovarian cancer were "sporadic"; that is, 56% (15/27) of affected women who carried a founder mutation had "no breast cancer in [their] family" (see Hartge et al. 1999, table 4). The same can be considered to have been observed in Israel by Abeliovich et al. (1997), once attention is properly focused on their "unselected" sample of just 24 carriers, of whom 58% (14) had no affected first-degree relatives. This general observation also applies to population-based samples of non-Ashkenazi populations. For example, in the United Kingdom, Peto et al. (1999) found that of the 30 women who were diagnosed with breast cancer at age ⩽45 years in whom a mutation in BRCA1 or BRCA2 was detected, 57% (17) had no family history of breast or ovarian cancer within three generations. In Australia, we have found that 72% (13/18) of BRCA1 or BRCA2 mutation-carrying cases diagnosed before age 40 years had no family history in the preceding two generations (Hopper et al. 1999).

Furthermore, only a small proportion of the typical women with "familial" breast cancer, defined as being affected *and* as having a family history of breast cancer, appear to have "hereditary" breast cancer, even in Ashkenazi Jews, in whom >2% of the population carries a mutation associated with a not-inconsiderable lifetime risk of ~50% (Struewing et al. 1997). The Washington study, in which there was a small bias toward living subjects having an excess of family history of breast cancer (Struewing et al. 1997), found that <10% of familial cases carried a founder mutation (Hartge et al. 1999), although it is conceivable that a smaller proportion may carry another "high-risk" mutation, either in BRCA1 or BRCA2 or, perhaps, another such gene whose normal function is to protect women from breast cancer. In our Australian population-based study of women with breast cancer at age <40 years, we detected a protein-truncating mutation in only 5% (6/120) of cases with at least one affected first- or second-degree relative

and in 9% (5/53) of cases with an affected first-degree relative, in a mutation screen covering between two-thirds and three-quarters of the coding regions of these two genes (Hopper et al. 1999, table 3).

Finally, the strong and highly significant effect that having had breast cancer has on the probability of being a mutation carrier could be used to derive an estimate of penetrance (i.e., age-specific cumulative risk of breast cancer), by using a case-control argument and appropriate population incidence rates and by taking into account the strong dependence of this effect on age, in which the odds ratio decreases from 10- to 2-fold across the four categories. In this regard, it is of interest that we found an average odds ratio of 9-fold for a set of protein-truncating mutations in BRCA1 and BRCA2 that cause early-onset breast cancer—and that this translates into a penetrance, until age 70 years, of just 40% when applied to Australian population rates (Hopper et al. 1999). Therefore, it is likely that a similar lifetime-penetrance estimate would apply to the founder mutations among U.S. Ashkenazi women, once the diminishing effect with age observed here has been counterbalanced by the ~30% higher underlying rates in the United States compared with Australia. Thus, population-based data on mutation carriers, such as those provided in some detail by Hartge and colleagues, are providing a new perspective on how genetic factors are evident in common diseases, challenging previous beliefs and language based on "monogenic" diseases (see Hopper et al. 1999).

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Electronic-Database Information

Accession numbers and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for BRCA1 [MIM 113705] and BRCA2 [MIM 600185])

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Response to the Letters from Hopper and Jenkins and Foulkes et al.

To the Editor:

These two thoughtful letters (Hopper and Jenkins 1999 [in this issue]; Foulkes et al. 1999 [in this issue]) illustrate some of the difficulties in drawing conclusions from the current body of data: even in very large studies, the number of subjects with breast or ovarian cancer in their families is small enough that different statistical models can yield quite different assessments of how likely a person is to be a mutation carrier. When the penetrance function has been securely established, probably the best model will be based on genetic inheritance (Berry et al. 1997) rather than on classification and regression trees (CART) (Breiman et al. 1984), multiple logistic regression (MLgR), or multiple linear regression (MLnR) (Wacholder 1986). We elected to explore the data with

CART, to build an "agnostic" model, close to the data, and added MLnR to try to separate the effects of various factors (Hartge et al. 1999). We chose not to use MLgR, to avoid distortion where data are sparse but projections are clinically relevant. For example, although the figure in the letter by Hopper et al. (1999) offers a clear qualitative depiction of the important factors, we caution that points on the graph depend heavily on choice of statistical model.

A word about MLgR: it can seriously misrepresent the data if the model is misspecified. Although MLgR is well suited to most problems in cancer epidemiology, in which the probability of disease developing is low for all exposure categories, it is not well suited here, where the probability of being a carrier, given personal and family history, can range from ~0% to ⩾20%. Under MLgR, an effect with an odds ratio of 2 raises the baseline risk of being a carrier, from 0.1% or 1% to ~0.2% or 2% but from 10% or 50% to ~18% or ~67%. Only with relatively high baseline risk would an odds ratio of 2 be an important factor in an individual's decision-making process.

The central conclusion from our volunteers remains that the carrier probabilities in those individuals with family history of breast or ovarian cancer are substantially lower than indicated by early published estimates.

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The Importance of a Family History of Breast Cancer in Predicting the Presence of a *BRCA* Mutation

To the Editor:

Hartge et al. (1999) describe the prevalence of the three founder Ashkenazi Jewish (AJ) mutations in *BRCA1* (MIM 113705) and *BRCA2* (MIM 600185) in 5,290 AJ volunteers from the Washington, DC, area. They report an overall mutation frequency of 2.3%, ranging from 1.2%, in those with no personal or first-degree-relative history of breast or ovarian cancer, to 50%, in women diagnosed with breast or ovarian cancer at age <40 years who had at least one first-degree relative with breast cancer diagnosed at age <50 years. The authors demonstrate, as we and others (Karp et al. 1997; Shattuck-Eidens et al. 1997; Fodor et al. 1998) have done, that, for the 297 women in their study with breast or ovarian cancer, the probability of carrying a *BRCA1* or *BRCA2* (*BRCA*) mutation decreases as age at diagnosis increases. Hartge et al. (1999, p. 965) state that, given age-at-onset information, "family history discriminated relatively little if the participant herself developed breast cancer, whereas, among other participants, family history best discriminated carriers from non-carriers." The age of the proband is clearly a powerful predictor of carrier probability, but our experience is that family history is an important determinant of the probability of a mutation, in both unaffected and affected women. Therefore, we reanalyzed Hartge et al.'s data, estimating relative risks of carrying a *BRCA* mutation for each age-at-diagnosis group (stratified by decade), in association with a first-degree-relative family history of breast cancer at any age ("positive family history") and in association with a first-degree-relative family history of at least one case of breast cancer diagnosed at age <50 years ("positive early-onset family history"). We analyzed affected and unaffected women separately. In affected women, the Mantel-Haenszel (M-H) odds ratio (OR), stratified by age at onset, for the association between a positive family history and the presence of a founder *BRCA* mutation, compared with a negative family history, was 2.6 (95% confidence interval [CI] 1.2–6.0, $P = .022$; ta-

Table 1

M-H OR for the Presence of a *BRCA* Mutation in Association with a Positive Family History of Breast Cancer, Stratified by Age or Age at Onset

	Hartge et al. (1999)						Warner et al. (1999): Affected Women		
	Affected Women			Unaffected Women					
	No. of			No. of			No. of		
	Noncarriers	Carriers[b]	OR [95%CI][c]	Noncarriers	Carriers[b]	OR [95%CI][c]	Noncarriers	Carriers[b]	OR [95%CI][c]
Age[a]									
<40 years									
FH × −	21	6	1.0	557	9	1.0	14	7	1.0
FH × +	4	3	2.6 [.5–15.0]	114	10	5.4 [2.4–12.5]	3	6	4.0 [.8–20.6]
40–49 years									
FH × −	71	6	1.0	874	14	1.0	80	13	1.0
FH × +	27	5	2.2 [.6–7.6]	215	9	2.6 [1.2–5.9]	31	10	2.0 [.8–5.0]
50–59 years									
FH × −	57	2	1.0	628	8	1.0	81	4	1.0
FH × +	19	4	6.0 [1.2–30.2]	169	6	2.8 [.9–7.8]	21	5	4.8 [1.3–17.8]
⩾60 years									
FH × −	46	1	1.0	611	4	1.0	95	2	1.0
FH × +	25	0	.6 [.0–15.5]	189	2	1.6 [.3–8.8]	39	1	1.2 [.1–13.9]
M-H			**2.6 [1.2–6.0]**			**3.1 [1.9–5.1]**			**2.6 [1.4–5.0]**
			P			*P*			*P*
Homogeneity			.50			.53			.63
Unity			.022			<.001			.004

[a] FH × = family history of breast cancer in any first-degree relative. The minus sign (−) indicates negative; the plus sign (+) indicates positive.
[b] Of a founder AJ *BRCA1* or *BRCA2* mutation.
[c] The logit estimate of the odds ratio was used when there was a zero cell.

ble 1, first "OR" column). For unaffected women, the M-H OR for the presence of a mutation in women with a positive family history was 3.1 (95% CI 1.9–5.1, $P < .001$; table 1, second "OR" column). For affected women, the M-H OR for the presence of a *BRCA* mutation in association with a positive early-onset family history was 4.4 (95% CI 1.7–11.4, $P = .003$; table 2, first column). This OR is greater than that observed in unaffected women with a positive early-onset family history (M-H OR 3.6, $P < .001$; table 2, second "OR" column). Thus, a family history of breast cancer is as predictive of the presence of a *BRCA* mutation in affected women as it is in unaffected women. The M-H OR for the unaffected and affected subgroups is similar and has overlapping CIs. The significance levels do differ, but this reflects the much larger size of the subgroup of unaffected women ($n = 4{,}993$; 94.4%) compared with those with breast or ovarian cancer ($n = 297$; 5.6%). In addition, a comparison of the strata-specific ORs in unaffected and affected women does not reveal a consistent pattern: none of the within-strata ORs differ statistically—the smallest *P* value is .38 (table 1, "OR" column 1 vs. column 2; table 2, "OR" column 1 vs. column 2).

To assess whether our reinterpretation of the Washington, DC, data set is valid, we performed the same analysis in 412 prevalent cases of breast cancer diagnosed in AJ women, ascertained between November 1, 1996, and May 31, 1998, in Toronto and Montreal (Warner et al. 1999). To compare exactly with the Washington, DC, study, we included only first-degree relatives with breast cancer in the analyses here. The definition of early-onset breast cancer was age at diagnosis of <50 years. The results are shown in tables 1 and 2, "OR" column 3. Notably, the M-H ORs seen in the Canadian study are identical to that observed in affected women in the Washington, DC, study: M-H OR 2.6 for the presence of a *BRCA* mutation in association with any first-degree-relative history of breast cancer and 4.4 for a positive early-onset family history. Thus, the findings from the Canadian study support our interpretation of the data published by Hartge et al. (1999) and lead us to question those authors' conclusion that the knowledge gained from knowing the family history of an affected person is "relatively small." The weight of evidence from clinical experience, from previously published work, and from their own study supports the conclusion that family history and age at diagnosis of breast

Table 2

M-H OR for the Presence of a *BRCA* Mutation in Association with a Positive Family History of Early-Onset Breast Cancer, Stratified by Age or Age at Onset

	Hartge et al. (1999)						Warner et al. (1999): Affected Women		
	Affected Women			Unaffected Women					
	No. of			No. of			No. of		
	Noncarriers	Carriers[b]	OR [95%CI][c]	Noncarriers	Carriers[b]	OR [95%CI][c]	Noncarriers	Carriers[b]	OR [95%CI][c]
Age[a]									
<40 years									
FH × 50 −	23	7	1.0	614	14	1.0	17	11	1.0
FH × 50 +	2	2	3.3 [.4–26.4]	57	5	3.9 [1.4–10.3]	0	2	7.6 [.3–173]
40–49 years									
FH × 50 −	92	8	1.0	1014	17	1.0	97	17	1.0
FH × 50 +	6	3	5.8 [1.4–23.9]	75	6	4.8 [2.0–11.4]	14	6	2.5 [.8–7.1]
50–59 years									
FH × 50 −	70	4	1.0	743	12	1.0	90	4	1.0
FH × 50 +	6	2	5.8 [1.0–32.6]	54	2	2.3 [.5–10.1]	12	5	9.4 [2.7–33.1]
⩾60 years									
FH × 50 −	61	1	1.0	734	5	1.0	122	2	1.0
FH × 50 +	10	0	2.0 [.1–51.2]	66	1	2.2 [.3–18.3]	12	1	5.1 [.548.0]
M-H			**4.4 [1.7–11.4]**			**3.6 [2.0–6.4]**			**4.4 [2.1–9.2]**
			P			*P*			*P*
Homogeneity			.81			.83			.40
Unity			.003			<.001			<.001

[a] FH × 50 = family history of breast cancer at age <50 years in any first-degree relative. The minus sign (−) indicates negative; the plus sign (+) indicates positive.

[b] Of a founder AJ *BRCA1* or *BRCA2* mutation.

[c] The logit estimate of the odds ratio was used when there was a zero cell.

cancer are both important factors in indicating the likely presence of a mutation in *BRCA1* or *BRCA2*.

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Electronic-Database Information

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DNA Sequence Variants of *p53:* Cancer and Aging

To the Editor:

p53 has a critical role in cell-cycle control. As such, it has been identified as an important target in human carcinogenesis. However, since human *p53* was cloned, ⩾10 DNA-sequence polymorphisms have been identified (Matlashewski et al. 1987; Weston and Godbold 1997). The codon 72 polymorphism (arginine/proline: G/C), the first to be described, has been the subject of ⩾31 epidemiological case-control studies that have explored a potential association with cancer (Olschwang et al. 1991; Weston et al. 1992, 1994, 1997; Zhang et al. 1992; Kawajiri et al. 1993; Birgander et al. 1995, 1996*b;* Jin et al. 1995; Själander et al. 1995*a,* 1996*a;* Wu et al. 1995; Murata et al. 1996; To-Figueras et al. 1996; Golovleva et al. 1997; Weston and Godbold 1997; Yung et al. 1997; Hayes et al. 1998; Helland et al. 1998; Hildesheim et al. 1998; Josefsson et al. 1998; Lanham et al. 1998; Minaguchi et al. 1998; Rosenthal et al. 1998; Storey et al. 1998; Tagawa et al. 1998; Wang-Gohrke et al. 1998). Although they err on the side of caution by citing Weston and Godbold (1997), Bonafè et al. (1999, p. 293), referring to the codon 72 polymorphism, state that "overall, the available data in the literature suggest that p53 variants may be considered as risk factors for some of the major neoplastic diseases in humans, such as lung, colorectal, breast and cervical cancer and are expected to affect survival." With respect to codon 72, we contend that this is probably not the case. In the available data, lung cancer is the subject of eight studies (Weston et al. 1992, 1994; Kawajiri et al. 1993; Birgander et al. 1995; Jin et al. 1995; Murata et al. 1996; To-Figueras et al. 1996; Tagawa et al. 1998). Three studies claim a statistically significant association: in one study, a subset analysis suggested a relationship in lung cancer cases diagnosed at age <53 years (Jin et al. 1995); in the other two, the allelic frequencies were almost identical (and the difference was not significant) between cases and controls (Kawajiri et al. [1993] observed a proline-allele frequency of .35 for controls and .36 for cases [n = 347 and 328, respectively]). Murata et al. (1996) observed a proline-allele frequency of .40 for controls and .35 for cases (n = 152 and 191, respectively), but associations of cancer risk were claimed on the basis of higher numbers of homozygotes (Kawajiri et al. 1993; Murata et al. 1996). One study (Kawajiri et al. 1993) implicated proline; the other (Murata et al. 1996), arginine.

Most recently, the relationship between cervical cancer, human papillomavirus (HPV) infection, and inheritance of the arginine allele has received considerable attention. The initial study, cited by Bonafè et al. (1999), showed a high degree of correlation between inheritance of the arginine allele and cervical cancer risk when HPV infection was present but not when it was absent (Storey et al. 1998). There are now eight studies of cervical cancer, but only one shows any association and seven are null (Hayes et al. 1998; Helland et al. 1998; Hildesheim et al. 1998; Josefsson et al. 1998; Lanham et al. 1998; Minaguchi et al. 1998; Rosenthal et al. 1998). There are five breast cancer studies (Kawajiri et al. 1993; Själander et al. 1996*a;* Weston et al. 1997; Helland et al. 1998; Wang-Gohrke et al. 1998); only one reached significance. Of the other published studies, a positive association has been claimed in 0/2 for nasopharyngeal cancer (Birgander et al. 1996*b;* Golovleva et al. 1997; Yung et al. 1997), 0/4 for colon cancer (Olschwang et al. 1991; Kawajiri et al. 1993; Jin et al. 1995; Själander et al. 1995*a*), 0/2 for bladder/urologic cancer (Kawajiri et al. 1993; Wu et al. 1995), 0/1 for acute myelogenous leukemia (Zhang et al. 1992), and 1/1 for stomach cancer (Kawajiri et al. 1993). Again, for stomach cancer, the allelic frequencies were not different for cases and controls, and an association of cancer risk with the arginine variant was claimed on the basis of higher numbers of arginine homozygotes in cases (Kawajiri et al. 1993; this report used the same control group noted above, and the proline-allele frequency in stomach cancer was .28 [n = 140]).

We contend, therefore, that the balance of the results of human molecular epidemiological association studies suggests that codon 72 allelism does not have an impact on human cancer risk. Specifically, at most, only 6 of 31 positive associations have been reported, and none have received consistent support in attempts to replicate them (Kawajiri et al. 1993; Jin et al. 1995; Murata et al. 1996; Själander et al. 1996*a;* Storey et al. 1998). Moreover, there is no obvious, plausible biological basis for an association of cancer risk with the codon 72 polymorphism. The impact of proline on the tertiary structure of a protein is disruption of α-helices. A proline residue resides at the monomeric position codon 71; therefore, at codon 72 there is no obvious consequence to proline.

Results were reported for *p53,* codon 72, genotype frequencies in healthy Italian centenarians and younger

Table 1

Simulated Relative-Risk Calculation for a Standard Population of 1,000 Persons

	No. of Cancer Deaths	No. of Survivors	Total
Proline-allele carriers	147	392	539
Arginine homozygotes	83	378	461
Total	230	770	1,000

NOTE.—On the basis of the control genotypic frequencies reported by Bonafè et al. (1999), a relative risk of 1.5, and a cancer death rate of 23% (WHO 1987; Lopez 1990), the frequency of proline-allele carriers in survivors is .509 (a reduction of 3.0%). If the relative risk is raised to 2.0, the frequency of proline-allele carriers in survivors is .491 (a reduction of 4.8%). This model addresses only a role for *p53* allelism in cancer as a cause of death. If limited to the major neoplasms indicated by Bonafè et al. (1999) (lung, colon, breast, and cervical), with a relative risk of 2.0, the frequency of proline-allele carriers in survivors is .526 (a reduction of only 1.3%). Also note that, if any association is limited to proline homozygotes, then, for a relative risk of 1.5, this fraction of the population would be expected to drop from .088 to .076 (a reduction of 1.2%). Bonafè et al. (1999), in fact, observed a drop of 2.0%, from .088 to .068.

controls (Bonafè et al. 1999). The expectation was of allelic winnowing in the case of a codon 72 variant associated with cancer susceptibility and subsequent survival. The frequency of proline-allele carriers was .539 in controls and .506 in centenarians, 3.3% lower (and the corresponding allelic frequency was 2.7% lower, but the difference was not significant). We conjecture that their comparison of centenarians (n = 176) with younger controls (n = 204) is too crude to detect the predicted allelic winnowing (Bonafè et al. 1999). First, the cause of death from cancer in the Italian population is ~23% (WHO 1987; Lopez 1990). Second, even though we do not agree that there is good evidence for an association between inheritance at codon 72 and cancer susceptibility, because of the molecular epidemiological data reviewed above, let us assume that the proline variant carries a relative risk of 1.5–2.0 (Kawajiri et al. 1993). On the basis of this assumption we might expect a reduction of 3.0%–4.8% in proline-allele carriers as the population ages (table 1 shows a simulated 2 × 2 table for a standard population of 1,000 persons, given a relative risk of 1.5 and a cancer death rate of 23% [WHO 1987; Lopez 1990]). In the letter by Bonafè et al. (1999), a reduction of 3.3% in the frequency of proline-allele carriers among centenarians was reported. This reduction is consistent with their hypothesis, although the authors suggest that it is not (Bonafè et al. 1999). Moreover, our corresponding projected reduction in allelic frequency is 2.8% for a relative risk of 1.5; Bonafè et al. (1999) observed a 2.7% reduction in frequency. However, to have, at a significance level of .05, 80% power to detect a 3.0%–4.8% reduction in the prevalence of proline-allele carriers, a minimum of 2,002–3,178 people would be needed, given a relative risk of 2.0; and 5,176–8,182 would be needed, given a relative risk of 1.5 (results of power calculations are given in table 2).

Notwithstanding these conclusions and observations, we believe that *p53* allelism is an important cancer-susceptibility factor but that it is more complex than that simply defined by the polymorphism at codon 72. A series of studies by a Swedish group (Beckman et al. 1994; Birgander et al. 1995, 1996*a,* 1996*b;* Själander et al. 1995*a,* 1995*b,* 1996*a,* 1996*b*) indicated that a constellation of three *p53* polymorphisms (intron 3, codon 72, and intron 6) constituted a haplotype predictive of increased breast cancer risk (odds ratio [OR] = 2.9, 95% confidence interval [CI] = 1.4–6.3 [Själander et al. 1996*a*]). Haplotypes were deduced on the basis of population frequencies of the individual polymorphisms. First, estimates of pairwise haplotype frequencies were calculated, and extended haplotype frequencies were deduced from this information. A subsequent study examined the same question but used a PCR-based method for physical determination of the haplotypes (Weston et al. 1997, 1998). The results of the latter study were consistent with those reported in the study by Själander et al. (1996*a*) (OR = 2.5, 95% CI = 1.3–4.8, in postmenopausal women) and further implicated a specific haplotype, designated "$p53^{1\text{-}2\text{-}1}$." More recently, a third study, estimating extended haplotypes of these three polymorphisms in a German population, provided results consistent with an elevated breast cancer risk associated with inheritance of $p53^{1\text{-}2\text{-}1}$ (OR = 2.0, 95% CI = 1.0–3.9 [Wang-Gohrke et al. 1998]). It is to be noted that this latest report has adopted an allelic nomenclature different from that developed by Beckman et al. (1994) and adopted by others (Själander et al. 1996*a;* Weston et al. 1997, 1998).

The balance of the results of human breast cancer studies of molecular epidemiological haplotype association—specifically, three positive associations in three

Table 2

Power Calculations

	No. of Centenarians Required for Hypothesis Testing When Frequency of Proline-Allele Carriers Is[a]	
OR	3.0%	4.8%
1.5	4,091	2,588
2.0	1,589	1,001

[a] Percentages are derived from calculations in table 1.

studies—suggests that inheritance of the $p53^{1\text{-}2\text{-}1}$ haplotype does have an impact on human breast cancer risk (Själander et al. 1996*a;* Wang-Gohrke et al. 1998; Weston et al. 1998). However, more research is needed to resolve this question. In our laboratory, we have adopted a complementary molecular epidemiological and basic-science approach. First, we are trying again to replicate the epidemiological studies of breast cancer, by using the PCR-based physical haplotype method in a population-based case-control study that has sufficient power. Second, we have derived normal human mammary cell strains with known haplotypes and plan to test their response to suspect breast carcinogens.

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p53 Codon 72 Polymorphism and Longevity: Additional Data on Centenarians from Continental Italy and Sardinia

To the Editor:

An extensive literature points out that p53 plays a pivotal role in cell cycle, apoptosis, and cell senescence and thus is crucial to a variety of physiological and pathological processes. These data support the view that p53 could be considered a gene that affects human longevity. In a previous letter (Bonafè et al. 1999) we tested the hypothesis that polymorphic variants of p53 have an impact on human longevity, by comparing p53 codon 72 allelic and genotypic frequency distributions between young people and centenarians. A nonsignificant difference emerged between the groups, and several explanations were offered. Following the reply letter of Sun et al. (in this issue), we would like to argue with some of their comments and to provide new data regarding centenarians from continental Italy and Sardinia. We disagree with the conclusion of Sun et al. that a consensus has been reached in the scientific community about the lack of any important role of p53 codon 72 in cancer susceptibility. First, several articles (not quoted by Sun et al.) still claim associations between codon 72 variants and lung cancer (Murata et al. 1998; Wang et al. 1999) and hepatocellular carcinoma (Yu et al. 1999). Second, in some cases, marginal differences regarding allelic and genotypic distributions between cases and controls have been found. In particular, Weston et al. (1997) reported *P* values of .054 and .07 when codon 72 Arg/Pro alleles and genotypes, respectively, were compared between Caucasian controls and patients affected with breast cancer. These authors failed to detect any differences in Mexican Americans or African Americans by use of case-control comparisons. These results cannot be considered conclusive evidence against an association, because the sample sizes analyzed were small (65 patients vs. 117 controls for Caucasians; 18 cases vs. 38 controls for Hispanics; 16 cases vs. 30 controls for African Americans). We doubt that samples of such size can be considered representative of Caucasian, Mexican, or African populations. Third, in several studies, important interactions among the codon 72 polymorphism and age (Murata et al. 1998), sex (Wang et al. 1999), environment, and other gene variants (Yu et al. 1999) have been reported. We tried to model this complex scenario (Bonafè et al. 1999), and methodologically new possible approaches have been proposed (De Benedictis et al. 1998; Yashin et al. 1998, 1999). Using experimental data on ApoB alleles in Italian subjects of different ages, including centenarians, we showed that the genotype relative risk (RR) of death can change with age, thus giving rise to unexpected nonmonotonic age-related trajectories. Comparable approaches to unraveling the possible effect of antagonistic pleiotropy on age-related genotypic changes during aging have been proposed (Toupance et al. 1997), with data on ACE genotypes in French centenarians. On the whole, unexpected results can be obtained by studying the genetics of centenarians. An understanding of the biological meaning of allele and genotype frequency distributions of centenarians requires an integrated approach that

Table 1

Allelic and Genotypic Frequencies of the p53 Codon 72 Polymorphism in Continental Italy

	No. (%) of	
	Controls (n = 611)	Centenarians (n = 394)
p53 Allele:[a]		
Pro	332 (27.8)	231 (28.3)
Arg	890 (72.2)	557 (71.7)
p53 Genotype:[b]		
Pro/Pro	49 (8.0)	29 (7.4)
Pro/Arg	234 (38.3)	173 (44.0)
Arg/Arg	328 (53.7)	192 (48.6)

NOTE.—Hardy-Weinberg equilibrium (HWE) of p53 genotypes was assessed by exact tests. Both groups were in HWE: younger controls, P = .53; and centenarians, P = .23. χ^2 Tests for comparison of allelic and genotypic distributions were performed by use of Monte Carlo algorithms implemented by means of the Statistical Product and Service Solutions (SPSS) package.

[a] χ^2 = 1.09, df 1, P = .29.

[b] χ^2 = 3.13, df 2, P = .20.

combines genetic and demographic data (Yashin et al. 1998, 1999). Calculations such as those proposed by Sun et al. may be inadequate, given that changes, with age, of RR for cancer (and death) have been reported for p53 variants (Weston et al. 1997). We disagree with the assumption that an RR of dying of 1.5–2 can be attributed to people carrying particular p53 codon 72 alleles. Sun et al. first strongly argue in favor of the hypothesis that codon 72 alleles are neutral, but then they assume this specific RR for Pro-allele carriers ("even though we do not agree there is a good evidence for an association between Codon 72 and cancer susceptibility, let us assume"). Because the power analysis performed by Sun et al. is based on this arbitrary assumption, their conclusions do not invalidate our interpretation of the data that we obtained in our study on the frequency of p53 variants in centenarians. We disagree with the conclusion of Sun et al. that the Pro/Arg sequence difference is functionally neutral. Recently, it has been demonstrated that codon 72 alleles differ biochemically and biologically in their ability to bind components of the transcriptional machinery, to activate transcription, to induce apoptosis, and to repress the transformation of primary cells (Thomas et al. 1999). We agree that haplotypes of codon 72 (*Bst*UI, exon 4), *Msp*I site (intron 6), and 16-bp insertion/deletion (intron 3) can help to detect associations in case-control studies regarding p53 polymorphisms and cancer susceptibility, but we disagree that the data available in the literature are conclusive. Indeed, in the three molecular epidemiology studies (Sjalander et al. 1996; Wang-Gohrke et al. 1998; Weston 1998 et al.) quoted by Sun et al. as proof of the involvement of a peculiar p53 haplotype in human cancer (breast), some inconsistencies should suggest a more cautious approach in the interpretation of the results. In particular, in the first study (Sjalander et al. 1996), the control sample was composed of a heterogeneous group of placental samples and blood samples from sex- and age-matched women. Moreover, a deficiency of heterozygotes and a slight deviation from Hardy-Weinberg equilibrium were found in the control group. These facts affect the reliability of p53 haplotype estimations. As stated by Sjalander et al., these data on genotypic combinations could "provide some idea" but did not offer definitive results concerning the role of a particular three-locus p53 haplotype in cancer susceptibility. In the second study (Wang-Gohrke et al. 1997), the results appear to be quite blunt (the *Bst*UI–16-bp–*Msp*I haplotype 1-2-1 increases in cases from .108 to .137, the 2-2-1 from 0 to .012) and apparently contradictory. Indeed, a significant difference between cases and controls, with regard to the 16-bp–*Msp*I 2-1 haplotype, appears to be present when a three-locus analysis is performed, but the same difference is not confirmed when a two-locus analysis is used. Thus, the biological role of p53 haplotypes in cancer susceptibility deserves further investigation. In analyses performed by Weston et al. (1997) the OR (odds ratio) estimates on "true" extended haplotypes are based on comparisons of 18 cases versus 38 controls and of 16 cases versus 30 controls. We think that ORs resulting from such small studies cannot provide reliable estimates of the RR of death for people carrying particular genotypes or haplotypes in Italian or other populations. Moreover, these three studies refer only to the risk for breast cancer. In our letter (Bonafè et al. 1999) the difference between the allele/genotype distributions of 176 centenarians and 204 controls was not statistically significant. However, Sun et al. made a number of speculations on the small differences between allele and

Table 2

Allelic and Genotypic Frequencies of the p53 Codon 72 Polymorphism in Sardinia

	No. (%) of	
	Controls (n = 92)	Centenarians (n = 110)
p53 Allele:[a]		
Pro	37 (20.1)	51 (23.2)
Arg	147 (79.9)	169 (76.8)
p53 Genotype:[b]		
Pro/Pro	4 (4.4)	4 (3.6)
Pro/Arg	29 (31.5)	43 (39.1)
Arg/Arg	59 (64.1)	63 (57.3)

NOTE.—Hardy-Weinberg equilibrium (HWE) of p53 genotypes was assessed by exact tests. Both groups were in HWE: younger controls, P = .75; and centenarians, P = .43. χ^2 tests for comparison of allelic and genotypic distributions were performed by use of Monte Carlo algorithms implemented by means of the Statistical Product and Service Solutions (SPSS) package.

[a] χ^2 = 0.55, df 1, P = .45.

[b] χ^2 = 1.25, df 2, P = .53.

genotype frequencies of young people versus centenarians. They claimed that these figures were consistent with their a priori hypothesis of an RR of 1.5–2 for death, for Pro/Pro (8.8% in controls vs. 6.8% in centenarians), Pro carriers (53.9% in controls vs. 50.6% in centenarians), and Pro alleles (31.4% in controls vs. 28.7% in centenarians). In association studies, it is not unreasonable to predict that such small, nonsignificant differences can vanish or reverse as the sample size increases or when replications are performed. Thus, we thought that the best approach was to provide experimental data by studying more subjects, either centenarians or controls, and to replicate the study in another population. Unfortunately, we were unable to study the 4,091 centenarians suggested by Sun et al. The reason was quite simple. According to the data of several laboratories, including our own, the prevalence of centenarians is ~80/1 million inhabitants in developed countries—such as Italy, Denmark, Finland, and Sweden, among others—where reliable age validation is possible. The actual number of centenarians living in Italy (which has ~57 million inhabitants) is thought to be ~4,500–5,000 (Capurso et al. 1997), considering the rapid increase of the oldest old in the population. We are not aware of anybody in the world capable of performing studies on 4,000 centenarians from an ethnically and/or geographically homogeneous population. This difficulty would be even higher if only a small minority of centenarians were relatively healthy. In table 1, new data are shown on allele and genotype frequency of p53 codon 72 polymorphisms in a total of 1,005 people. An additional 218 centenarians and 407 controls have been included with respect to the data reported elsewhere (Bonafè et al. 1999). The data reported here confirm, in a larger sample, that no significant difference is detectable between centenarians and young controls.

With the various possible biases inherent in association studies, replications of studies in other ethnically and/or geographically different populations can help us to better understand the obtained results. Thus, we thought it worthwhile to study the allelic and genotypic distributions of p53 codon 72 in Sardinian centenarians and appropriate younger controls (Deiana et al., in press). The data regarding Sardinian centenarians are reported in table 2. In Sardinian controls, the frequencies of p53 Pro and Arg alleles are 20.1% and 79.8%, respectively. This frequency is significantly different from that found in controls from continental Italy (χ^2 = 4.11, df 1, P = .042). Despite this ethnic difference, again, no statistically significant difference was found between Sardinian centenarians and Sardinian controls. The difference between the two groups, with regard to the frequency of the Pro 72 allele, was ~3% in favor of centenarians. Moreover, the Pro 72 allele carriers were slightly increased in Sardinian centenarians (42.7% in centenarians vs. 35.9% in controls), and this trend is similar to that found in centenarians from continental Italy (table 1). We consider these data on Sardinian centenarians to be additional evidence that both p53 codon 72 alleles are equally compatible with extreme longevity.

In conclusion, we consider the study of the genetics of extreme longevity to be worthwhile, even if some important effects can be missed by the limited size of the samples that can reasonably be recruited. These limitations are frequently encountered even in other types of association studies, including cancer studies (Weston et al. 1997). In any case, the study of p53 haplotypes related to cancer susceptibility can be of considerable help in disentangling this difficult topic. We are following this approach, and we hope that new data will emerge soon.

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Elevated Frequency and Allelic Heterogeneity of Congenital Nephrotic Syndrome, Finnish Type, in the Old Order Mennonites

To the Editor:

Congenital nephrotic syndrome is a clinically and genetically heterogeneous disorder of the glomerular filtration barrier, characterized by massive proteinuria at or shortly after birth. Although nephrotic syndromes encompass a heterogeneous group of disorders, congenital nephrotic syndrome of the Finnish type (NPHS1 [MIM 256300]) is a distinct clinical entity. NPHS1 has an autosomal recessive mode of inheritance and is generally rare—except in Finland, where the incidence is ~1/10,000 live births (Norio 1966). Nephrin, a putative transmembrane protein belonging to the immunoglobulin family of cell-adhesion molecules, has been identified as the gene mutated in NPHS1, with loss-of-function deletion and missense mutations identified in Finnish and other white patients with NPHS1 (Kestilä et al. 1998; Lenkkeri et al. 1999). Several cases of NPHS1 have also been observed in other populations, with or without direct evidence of Finnish ancestry (Fuchshuber et al. 1996). On the basis of limited haplotype analysis, it has been argued that some cases of non-Finnish NPHS1 may have a common origin in Finland (Männikkö et al. 1996).

We observed a high incidence of NPHS1 among the Old Order Mennonites in Lancaster County, Pennsylvania. In particular, we identified 26 cases of NPHS1, dating from the 1950s, in a very large inbred Mennonite kindred (population). Interestingly, all but one of the cases of NPHS1 in Mennonites occurred in a subgroup known as the "Groffdale Conference" Mennonites. The

Groffdale Conference Mennonites formed as a result of a schism in the Weaverland Conference Mennonites of Lancaster County in 1927. The separation between the two groups was based on differing views regarding acculturation to American thought and society. The more conservative group formed the Groffdale Conference, whereas the more progressive members continued in the Weaverland Conference. At present, the populations and birth rates of the two conferences are approximately equal.

We estimated the incidence of NPHS1 among the Groffdale Conference to be .002 or 1/500 live births, on the basis of the complete ascertainment of eight cases among 4,062 births during the period 1985–94. This incidence is 20 times greater than that observed in Finland and predicts that ~8% of Groffdale Mennonites are carriers of the NPHS1-causing allele. The ancestors of this Old Order group immigrated from Switzerland to Lancaster County during the early 18th century, and no explicit Finnish ancestry is known. In this study, we (1) confirm the role of nephrin in NPHS1, (2) show that a major mutation is shared by families with NPHS1 that are in the Groffdale Conference, and (3) show that this mutation is most likely of recent origin—uncovered by inbreeding and amplified by genetic drift. Our data also suggest that the major Mennonite mutation most likely predated the Weaverland-Groffdale split, since one Weaverland NPHS1 proband is a double heterozygote with one copy of the major nephrin mutation as well as a second novel mutation, possibly contributed through a non-Mennonite lineage.

We initially ascertained four families, in each case through a proband with NPHS1. The four families had 8 obligate carrier parents and 11 unaffected offspring. Only one of the probands from these four families was alive during the study. Subsequent to our analysis of these four families, one additional family—which included a second surviving proband, parents, and paternal grandparents—was ascertained. After our mutation-detection studies were completed, three additional families with NPHS1 agreed to participate.

One of the two living Mennonite probands with NPHS1 (6986) was less-severely affected than the other patients. This patient underwent nephrectomy at age 18 mo, followed by nightly peritoneal dialysis and a renal transplant at age 3.5 years. Prior to nephrectomy, this child had an array of life-threatening problems, including recurrent renal-vein thrombosis, severe hypertension, encephalitis, hypothyroidism, refractory anemia, hypercholesterolemia, edema, ascites, and malnutrition. The second surviving Mennonite child was 30 mo of age at the time of our study. She had minimal medical interventions until age 2 years, at which time she was started on the Finnish protocol of enalapril, thyroid replacement, vitamin D, and erythropoetin (Guez et al. 1998). Within 4 wk of the start of enalapril therapy, her serum albumin increased from 0.6 to 2.1 g/dl and her generalized edema resolved. If she survives until she obtains a weight of 10–12 kg, renal transplantation will be reconsidered.

For genetic analysis, we obtained, with informed consent, either peripheral blood samples or buccal swabs from members of the eight families. Genomic DNA was extracted according to standard procedures. No pathology samples were available from the deceased affected individuals, to perform additional analyses. Microsatellite-marker alleles were amplified and genotyped via standard PCR methods (Puffenberger et al. 1994). Allele sizes for all markers (except D19S608, D19S609, and D19S610) were determined in comparison with CEPH individual 1347-02; allele numbers for D19S608, D19S609, and D19S610 were assigned arbitrarily. To determine the genomic structure of nephrin, we used the "BLAST 2-sequences" option of the program BLAST (Tatusova et al. 1999), to align nephrin cDNA sequence and overlapping genomic sequence from cosmid R33502 (GenBank AFO35835 and AC002133). The gene consists of 29 exons and spans ~26 kb; primer sequences, from the program Oligo, version 4.0, were designed for PCR amplification of all 29 exons in 19 reactions (table 1). For mutation analysis, nephrin exons were PCR amplified from one patient (3898), the patient's father (3833), and a CEPH control DNA. After PCR amplification, products were sequenced on an ABI377 sequencer (PE Biosystems), with the use of standard dye-terminator chemistry.

We expected that any NPHS1 candidate locus will have a unique haplotype in Mennonites, which is homozygous in affected individuals, heterozygous in the 8 obligate heterozygote parents, and not homozygous in the 11 unaffected siblings (fig. 1 shows all individuals but the unaffected siblings). Using 10 microsatellite markers, Männikkö et al. (1995) mapped NPHS1 to chromosome 19q13.1 by linkage and linkage-disequilibrium analysis in Finnish patients. We genotyped these same markers in the 20 available individuals and identified a common haplotype (table 2) for five markers in the 3.5-cM interval D19S609–D19S220, satisfying the above criteria and implicating nephrin in Mennonite NPHS1. It is unclear whether any of the Finnish NPHS1 haplotypes are in common with Mennonite NPHS1 haplotypes, since Kestilä et al. (1998) have not published actual allele sizes at the marker loci studied.

Nucleotide sequencing of all nephrin exons (table 1) from one proband revealed the deletion of one nucleotide within exon 12 (1481delC), which segregated with the NPHS1 haplotypes identified. In addition to the families shown in figure 1, six parents from three additional Mennonite sibships with NPHS1 were genotyped for the 1481delC mutation; all were heterozygous. This deletion

Table 1

Oligonucleotide Primers for Amplification of NPHS1 Exons

Exon(s)	Primer Sequences	Annealing Temperature (°C)	Product Size (bp)
1, 2	Forward: AGACGCAAGGTGGCTGGCAGCG Reverse: TTCCGCTGGTGGCTGAGGGTC	60	474
3, 4	Forward: AGCGGAGCTGCGGCCCTGACT Reverse: CCTTCCCACTCCAGAGGCTTCA	63	520
5	Forward: TCGCACCCACCCTGAGGACTTC Reverse: ATGCTTGCATCCCTGGGGTCTG	60	252
6	Forward: CCCCACCTCTTCTCCCTGACT Reverse: ATCCCCCCACACCCCCCAGTG	60	246
7, 8	Forward: GGAAAGAGTGGATGGGCTACT Reverse: CTCTGAGGCACAGACCGACAG	60	486
9	Forward: TCTGGGCTGGTCTGTGAGAAA Reverse: TCAGCCCCCTCCATGCTCAGA	55	264
10, 11	Forward: GTGCTGCAGTCCCCACGTCTG Reverse: CATTCCTGGCCACCCCCATAG	60	435
12	Forward: TGCTGATGAGAGTGCTTCTCC Reverse: CACCCCAGGCTCCGCCCAGTC	60	467
13	Forward: CTGGGCGGAGCCTGGGGTGCA Reverse: CAGAGGCTGGAGAGGCACTAG	60	308
14	Forward: ATCCCTCCCCTCTCTGGTCTG Reverse: AAGGTAAGACCCAAGGAGTAG	60	356
15, 16	Forward: AACCTTAAACCCCGTCGTGAC Reverse: CAATGAGGAGACTCCACAATG	60	543
17	Forward: AACCTTCCTGGAACCCCTAAG Reverse: CACTCCCAAGGAACTCACAGT	57	300
18, 19	Forward: GTGATGGATCTGGGGCTAGAC Reverse: AGGGACTCAGGGAGGGGAAGT	59	558
20	Forward: GATAGATAGGCAGACGGTTAC Reverse: AACTCCATCCTCACACATACA	55	382
21, 22	Forward: TCTTCTGGAATTCTTTTGTAT Reverse: TACACATCCTCTGAGGAATAC	55[a]	474
23	Forward: ACCAGTTCCCATGAATCTAATA Reverse: GTCGTTAAGCAGCTGTGACTAC	55	262
24–26	Forward: CAGCCTGTTGTCTGGGATTC Reverse: GCTTCAGTCGCCGTCGGTGC	60	610
27, 28	Forward: TCCGGGCACAGTGGGGTCAGAG Reverse: GCAGCTAGCTGGCCCTAACTAA	59	454
29	Forward: TCCATGGTTCTAACTCACTT Reverse: GCACTTAGGGACCCTCAGAATAG	60	580

[a] Requires 10% DMSO.

mutation is novel (Kestilä et al. 1998) and leads to a frameshift and premature truncation of the protein, to 547 residues. Nephrin is a 1,241-residue protein predicted to be a cell-adhesion receptor and/or a signaling protein. Its predicted structure includes eight Ig-like domains and one fibronectin-like domain, adjacent to a transmembrane region. The truncation caused by the Mennonite mutation occurs well before the putative transmembrane domain (residues 1059–1068); therefore, it follows that 1481delC is very likely a null allele and that homozygotes have no functional protein.

Subsequent to the identification of 1481delC, we ascertained the only other living Mennonite proband (6986 [fig. 2]) and his immediate family. Interestingly, this patient was found to be heterozygous for 1481delC and inherited the allele maternally. After nephrin sequence analysis of this patient and his father, we detected a second frameshift mutation, 3250delG, for which both were heterozygous. Genealogical analysis revealed that proband 6986 has a partially non-Mennonite lineage; his maternal great-grandmother married into the Mennonite kindred (fig. 2). We were able to trace the second mutation, 3250delG, to the patient's paternal grandfather by DNA analysis. Since the proband's non-Mennonite great-grandmother is deceased, we could not establish whether she contributed this mutation or whether the occurrence of 3250delG is a new mutation in the proband's grandfather. Nonetheless, we identified a second unique mutation, which also results in premature truncation of nephrin protein, resulting in a

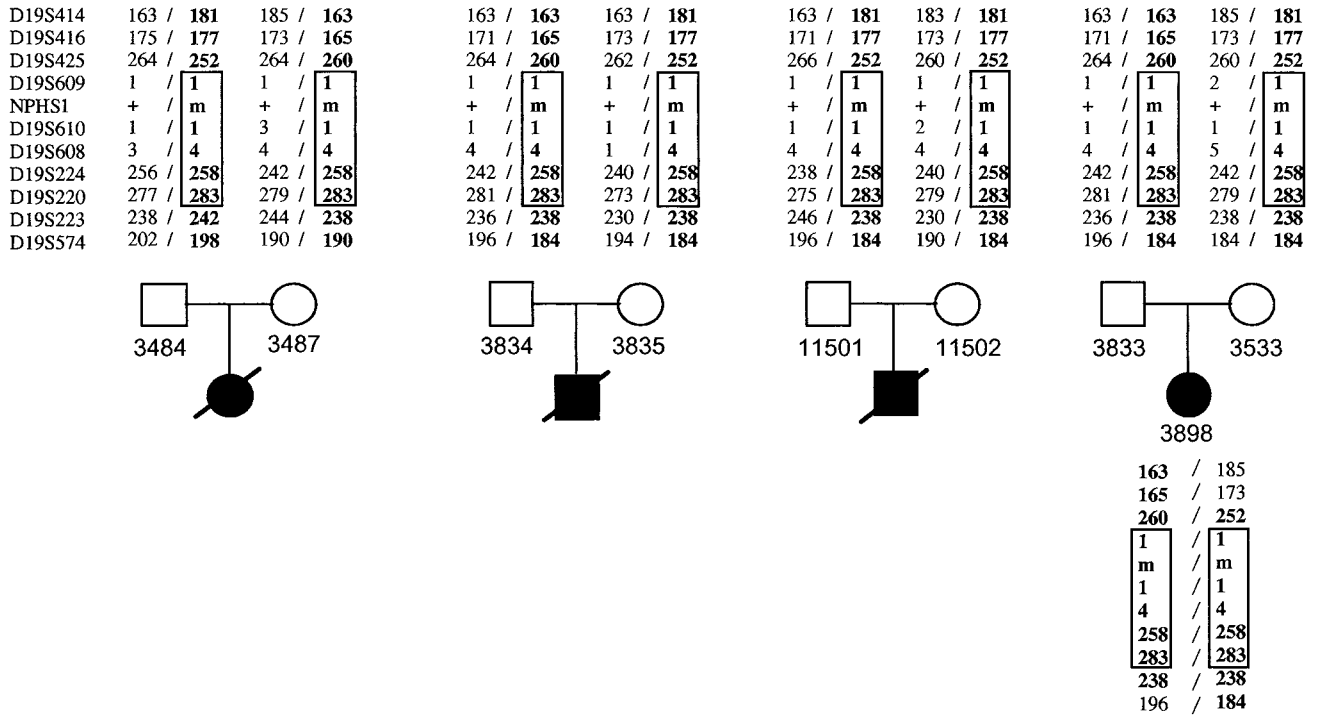

Figure 1 Haplotype analysis of four Mennonite nuclear families with NPHS1. Chromosome 19q13 haplotypes, of the markers shown in table 2, of NPHS1-transmitting parents and homozygosity for a haplotype in proband 3898 are shown. None of the 11 unaffected siblings from the four families were homozygous for the identified haplotype (data not shown). The common mutation, 1481delC, segregates with the haplotype designated in boldface.

1,141-residue protein. According to the predicted structure of the protein, it is likely that the 1,141-residue protein inserts into the membrane but lacks the complete cytosolic domain. Thus, some residual nephrin function may exist and may explain the less-severe phenotype of this patient.

Our results confirm the role of nephrin in NPHS1 and allelic heterogeneity within the Mennonites. NPHS1 is very rare throughout the world, and its low incidence is likely under mutation-selection balance; for a recessive lethal mutation, the incidence would equal its de novo mutation rate per generation, which has a mean value of $\sim 10^{-6}$ (Cavalli-Sforza and Bodmer 1971). The ~1/10,000 incidence in Finland is two orders of magnitude larger and is likely to have increased by genetic drift and founder effects during the past 100 generations (2,000 years) of Finnish history. Moreover, the occurrence of at least three distinct mutations, each on a specific marker haplotype with estimated frequencies of 0.78%, 0.16%, and 0.06% (Kestilä et al. 1998), suggests that the age of the more frequent mutation is not recent. This is corroborated by the observation of linkage disequilibrium, over a small distance (~150 kb), for the major Finnish NPHS1 mutation (Kestilä et al. 1998). As we have shown here, these effects are further amplified in the Old Order Mennonites, which have a much more recent history and a much smaller effective population size.

In this study, we have estimated the Mennonite NPHS1 incidence at ~1/500 live births, or an overall 20-fold increase compared with the Finnish incidence. This large increase is undoubtedly from genetic drift and founder effects (Chakravarti and Chakraborty 1978), consistent with the finding of a major common mutation (1481delC) in a population with an estimated effective number of ~200 (A. Chakravarti and E. G. Puffenberger, unpublished data). We argue that the 1481delC mutation is likely of very recent origin. Table 1 shows that the core NPHS1 haplotype, on which the 1481delC mutation is found, is shared by all eight mutant chromosomes and is $\geq$3.5 cM; however, haplotypes 1 and 4 (five copies total) and haplotypes 2 and 3 (three copies total) each share the longer D19S414–D19S220 haplotype, of 10 cM. Therefore, the average time to origin of the common mutation in the Mennonites is ~1/0.1 morgans, or ~10 generations. It is possible either that this was a rare mutation in the European Mennonite groups and that it expanded in the United States or that the mutation is of recent U.S. origin.

As mentioned above, the distribution of NPHS1 is not random within the Old Order Mennonite community

Table 2

Mennonite NPHS1 Mutant-Allele–Carrying Haplotypes

Marker[a]	Intermarker Distance (cM)[b]	Haplotype[c]			
D19S414	4.9	181	163	163	181
D19S416	.6	177	165	165	177
D19S425	1.0	252	260	260	252
D19S609		1	1	1	1
D19S610	3.0	1	1	1	1
D19S608		4	4	4	4
D19S224	.5	258	258	258	258
D19S220	2.2	283	283	283	283
D19S223	5.6	238	238	238	242
D19S574		184	184	190	198

[a] Markers in the segment common to all haplotypes and that implicate nephrin in NPHS1 span a genetic distance of ⩾3.5 cM and are underlined.

[b] Between the marker shown and that immediately below (except for "3.0," which denotes the distance spanned by the four markers underlined).

[c] The boxed portion indicates a common haplotype segment shared by all parents of the affected individuals whom we studied. The number of copies is four, two, one, and one, respectively.

and is largely restricted to the Groffdale Conference. The Groffdale and Weaverland Conferences share very recent ancestry, yet the distribution and allelic spectrum of NPHS1 mutations are not equal within these two groups. At present, only 1 case of NPHS1 has been observed in the Weaverland Mennonites, whereas 25 cases have been identified in the Groffdale Mennonites since the 1950s. We have found that the single Weaverland Conference patient with NPHS1 (6986) is a compound heterozygote for the major Mennonite mutation, 1481delC (same core haplotype as in table 2), and a second mutation, 3250delG. Our analysis shows that 1481delC is the only mutation segregating in seven nuclear families from the Groffdale Conference (fig. 2). It is interesting that the origin of 3250delG is non-Mennonite, since it is inherited through the non-Mennonite ancestors of proband 6986. These data emphasize the rarity of NPHS1 mutations in most populations, identified here among non-Mennonites only when combined with a common Mennonite mutation. Moreover, the concentration of 1481delC in the Groffdale Mennonites suggests that NPHS1 occurs at an incidence much higher than 1/500. The incidence within each conference cannot as yet be determined with any greater precision.

The accumulation of NPHS1 in the Groffdale Conference and the rarity of 1481delC in the Weaverland Conference can be explained in multiple ways. First, a subfounder effect might explain the high incidence within the Groffdale group. However, genealogical and mutational analyses do not support this hypothesis, since there is no common ancestor for all carriers of NPHS1 who were born after the 1927 schism and 1481delC is present in the Weaverland Mennonite population. Second, 1481delC may have arisen in the Groffdale Mennonites and may have been reintroduced into the Weaverland Conference. Since marriage between the groups is extremely rare, it is unlikely, although not impossible, that the mutation was contributed to the Weaverland Conference after the 1927 split. An alternative and simpler explanation is that 1481delC arose prior to the split and that it has increased in frequency through genetic drift. The formation of the Groffdale Conference by family groups wishing to preserve a simpler lifestyle may have led, by chance, to a relative increase in the mutant-allele frequency in one conference compared with the other.

It is surprising that a recent mutation has achieved such a high frequency in a short time. This is likely a result of the intense inbreeding that occurs within the Old Order Mennonites in each generation—not from high rates of social consanguinity but from the limited ancestral pool and the sharing of multiple common ancestors between any two individuals. We have established a genealogical database of >7,500 individuals from the Old Order Mennonites that allows direct calculation of kinship coefficients between any two individuals, equivalently the inbreeding coefficient for their offspring. We studied 12 pairs of parents with complete genealogies and with an offspring affected with NPHS1; the average kinship coefficient was .020 (range .010–.037), corresponding to a relationship between first cousins once removed and second cousins. For the four pairs of parents that we report here, the average kinship coefficient was .017 (range .014–.019). These

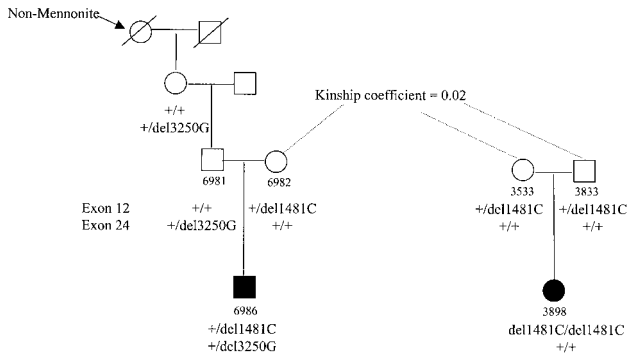

Figure 2 Genealogy of two nephrin-deletion mutations. The single Mennonite proband who is doubly heterozygous and who inherited the common Mennonite mutation and a second mutation from a non-Mennonite ancestor is shown. The kinship coefficient of the ancestors shown clarifies that this proband is closely related to other Mennonites with NPHS1.

rates are relevant for most individuals in the last several generations. Consequently, any de novo mutation occurring in the recent past has a high chance of becoming homozygous, through inbreeding, in the Mennonites. This is in contrast to outbred populations, in which, with random mating, rare mutants become homozygous only rarely.

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BLAST, http://www.ncbi.nlm.nih.gov/BLAST (for alignment of sequences)

GenBank, http://www.ncbi.nlm.nih.gov/Web/Search (for nephrin cDNA [accession number AF035835] and for cosmid R33502 [accession number AC002133])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for NPHS1 [MIM 256300])

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Complete Inactivation of the TSC2 Gene Leads to Formation of Hamartomas

To the Editor:

Tuberous sclerosis complex (TSC [MIM 191092]), a dominantly inherited disease with a prevalence of 1/6,700 (Sampson et al. 1989; Osborne et al. 1991; Ahlsen et al. 1994) is characterized by hamartomas found in almost every organ system and by malignancy in some organ systems. Two genetic loci have been identified: the TSC1 gene, on chromosome 9q34.3, and TSC2, on chromosome 16p13.3 (The European Chromosome 16 Tuberous Sclerosis Consortium 1993; van Slegtenhorst et al. 1997). A tumor-suppressor function has been suggested for TSC2 by loss of heterozygosity (LOH) in hamartomas (Green et al. 1994; Henske et al. 1995, 1996; Carbonara et al. 1996; Sepp et al. 1996). Thus far, no reports have demonstrated mutations in both alleles of the TSC2 gene in hamartomas in humans. Further evi-

Table 1

Summary of LOH Studies

Patient and Tumor Sample	LOH Findings for Germline Mutation								
	D16S525	Exon 18 (dl C2070)	Exon 24 (insC 2779)	Exon 30 (dup3611-27)	Exon 36 (dl4770-2)	Exon 37 (A4859T)	Exon 40 (EcoRV)	KG8	D16S665
HOU23-01:									
1T	LOH	…	LOH	…	…	…	LOH	NI	LOH
rT1	LOH	…	LOH	…	…	…	LOH	NI	LOH
rT2	LOH	…	LOH	…	…	…	LOH	NI	LOH
rT3	H	…	H	…	…	…	H	NI	H
rT4	H	…	H	…	…	…	H	NI	H
rT5	LOH	…	LOH	…	…	…	LOH	NI	LOH
rT6	LOH	…	LOH	…	…	…	LOH	NI	LOH
HOU23-03:									
T	H	…	H	…	…	…	H	NI	H
TS94-104:									
T1	NI	LOH	…	…	…	…	NI	LOH	LOH
T2	NI	LOH	…	…	…	…	NI	LOH	LOH
T3	NI	LOH	…	…	…	…	NI	LOH	LOH
T4	NI	LOH	…	…	…	…	NI	LOH	LOH
T5	NI	LOH	…	…	…	…	NI	LOH	LOH
T6	NI	LOH	…	…	…	…	NI	LOH	LOH
T7	NI	LOH	…	…	…	…	NI	LOH	LOH
T8	NI	LOH	…	…	…	…	NI	LOH	LOH
T9	NI	LOH	…	…	…	…	NI	LOH	LOH
T10	NI	LOH	…	…	…	…	NI	LOH	LOH
T11	NI	LOH	…	…	…	…	NI	LOH	LOH
T12	NI	LOH	…	…	…	…	NI	LOH	LOH
T13	NI	LOH	…	…	…	…	NI	LOH	LOH
T14	NI	LOH	…	…	…	…	NI	LOH	LOH
TS93-41:									
FA1	H	…	…	H	…	…	NI	H	NI
FA2	H	…	…	H	…	…	NI	H	NI
FA3	H	…	…	H	…	…	NI	H	NI
TS93-14:									
T	H	…	…	…	H	…	H	H	H
TS94-53:									
T	NI	…	…	…	…	H	H	NI	H

Note.—NI = noninformative; ellipsis (…) = not applicable; and H = maintainance of heterozygosity.

dence of a tumor-suppressor function for TSC2 comes from the Eker rat, a naturally occurring model of tumorigenesis. The Eker rat has been determined to have a germline mutation in the Tsc2 gene (the homolog of the TSC2 gene in humans) (Yeung et al. 1994; Kobayashi et al. 1995; Kubo et al. 1995). LOH and second somatic hit studies of the Tsc2 gene have been reported in 33% of spontaneous or chemically induced Eker rat renal-cell carcinoma (RCC) lines (Kobayashi et al. 1997). The mechanism for cell transformation in humans is expected to be different from that in rats, because RCCs are detectable in 100% of Eker rats by age 1 year, whereas only a small percentage (2.5%) of humans with TSC develop RCCs (Eker et al. 1981; Bjornsson et al. 1996; Cook et al. 1996; Al-Saleem et al. 1998). We collected a variety of hamartomas—angiomyolipomas (AMLs) from three patients, cortical tubers from two patients, and facial angiofibromas (FAs) from one patient—from patients in whom the germline TSC2 mutation had been identified (Au et al. 1998). These tissues were tested for the second hit by Southern blotting, with the TSC2 gene as a probe, and by LOH studies of the TSC2 gene and surrounding markers. The second hit for the TSC2 gene was detected in AMLs from two independent patients and in FAs from another patient, providing direct evidence that mutations in both alleles are required for tumorigenesis in humans affected by TSC (Knudson 1971).

The patients in this study have been described elsewhere (Au et al. 1998). All were diagnosed with TSC according to standard criteria by Roach et al. (1998). Informed consent approved by the institutional review boards at The University of Texas Medical School–Houston, The University of Texas Southwestern Medical School, and the Scottish Rite Hospital in Dallas was obtained. Tissue samples collected included 6 AMLs from the right kidney and 1 AML from the left kidney of patient HOU23-01, 1 AML from patient HOU23-03,

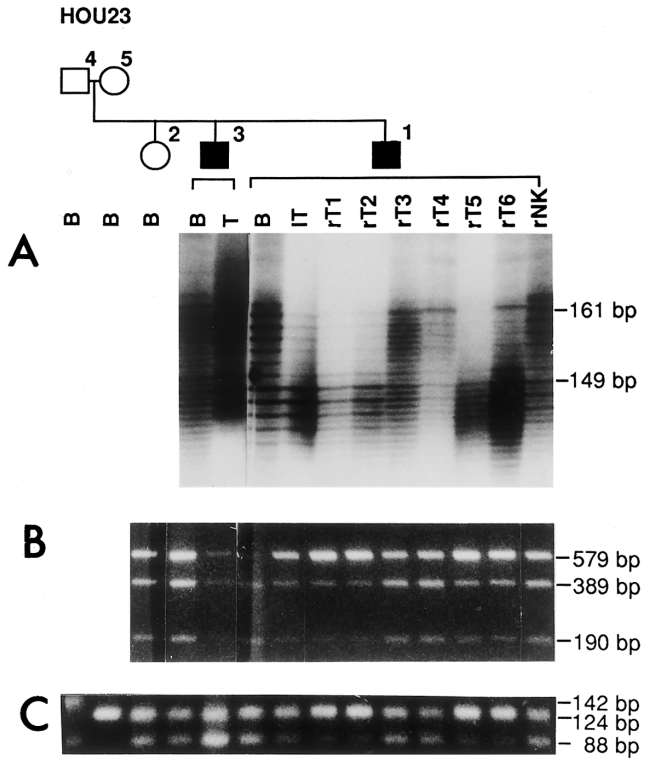

Figure 1 Testing of DNA samples from family HOU23, including members HOU23-04 (unaffected father), HOU23-05 (unaffected mother), HOU23-02 (unaffected child), HOU23-03 (affected child), and HOU23-01 (affected child), as depicted on pedigree shown above gels. The family represents an example of germline mosaicism, details of which are reported elsewhere (Rose et al. 1999). Individual lanes are labeled to indicate tissue from which DNA was extracted: B = blood lymphocytes, T = AML sample from HOU23-03, lT = left kidney AML, rT1–rT6 = six individual right-kidney AML samples, and rNK = right-kidney normal tissue from HOU23-01. Decrease in intensity of bands indicating loss of the wild-type TSC2 allele in some cells of the AMLs from patient HOU23-01 are observed in *A–C*. *A,* Marker D16S525 is located ~95 kb telomeric to the 5′ end of TSC2. Both HOU23-01 and HOU23-03 are heterozygous, with alleles of 149 bp and 161 bp present. The 161-bp allele was significantly reduced in the lT, rT1, rT2, rT5, and rT6 of HOU23-01, whereas the 149-bp and 161-bp alleles were of equal intensity in rT3 or rT4 of HOU23-01 and in the AML (T) from HOU23-03. *B,* Patients were informative for the *Eco*RV polymorphism in exon 40 of TSC2, with heterozygotes showing three bands at 579, 389, and 190 bp, respectively. Decrease in the intensity of the wild-type allele was found in the same AML DNAs (lT, rT1, rT2, rT5, and rT6) as in *A,* indicating LOH for the wild-type allele in some cells from which the DNA was extracted. *C,* The marker D16S665 is located ~75 kb from the 3′ end of TSC2. The father (HOU23-04) is heterozygous for the marker, with alleles of 142 and 88 bp, and the mother is homozygous for the 124-bp allele. The results showed the same five AMLs (lT, rT1, rT2, rT5, and rT6) with decreased intensity of the paternal 88-bp allele, while retaining the maternal 124-bp allele. The paternal 142-bp allele is not inherited by any of the offspring in the family.

14 AMLs from patient TS94-104, 3 FAs from patient TS93-41, and 1 cortical tuber each from patients TS93-14 and TS94-53.

DNA from the blood lymphocytes of the patients was prepared as previously described. Small samples of tissue were removed from the well-circumscribed tumors at the time of surgery. Fresh tumor tissue weighing ~0.2 g (or the whole tumor, if it was <0.2 g) was dissected, minced, and rinsed with phosphate-buffered saline to remove red blood cells. The minced tissue was digested with 1 mg proteinase K/ml in 10 ml of lysis buffer, with remaining processing being the same as that for lymphocyte DNA.

Individual TSC2 gene exons containing the germline mutation were amplified by polymerase chain reaction (PCR) from lymphocytic DNA and from the tumor DNA, as described elsewhere (Au et al. 1998). The intragenic polymorphic *Eco*RV marker in exon 40 was tested in informative cases (Au et al. 1997). Chromosomal markers (D16S525, KG8, and D16S665) flanking the TSC2 gene were tested as described elsewhere (Peral et al. 1994; Shen et al. 1994; Snarey et al. 1994).

Southern analyses were performed as described elsewhere (Au et al. 1997). The TSC2 gene fragments were identified sequentially, with different segments of the TSC2 cDNA used as probes. One probe, consisting of nucleotides 1–1197 of the TSC2 cDNA, detects two adjacent *Bam*HI fragments of 15 kb (exons 1–8) and 7 kb (exons 9–14) and a 15-kb *Hin*dIII fragment (exons 1–11) of the TSC2 gene. Another probe used for verification, consisting of nucleotides 1369–2689 of the TSC2 cDNA, detects two adjacent *Bam*HI fragments—of 7 kb (exons 9–14) and 14 kb (exons 15–24)—and a 15-kb *Hin*dIII fragment.

In all cases, the TSC2 allele with the germline mutation was retained in the tumor DNA (table 1). When flanking markers D16S525, KG8, and D16S665 and an intragenic marker, TSC2 exon 40 *Eco*RV, were used, LOH was not detected in DNA from the one AML from patient HOU23-03, from the three FAs from patient TS93-41, or from the two cortical tubers from patients TS93-14 and TS94-53 (data not shown).

Seven samples of tissue from AMLs were available from patient HOU23-01: six from the right kidney and one from the left kidney. The TSC2 allele carrying the germline mutation was retained in all seven tumors (table 1). Five of the seven AMLs (four from the right and one from the left) showed decreased intensity for one allele with markers D16S525, TSC2 exon 40 *Eco*RV, and D16S665, indicating LOH for some cells from those samples (fig. 1). The alleles with decreased intensity for the markers were the same in all five tumors (lT, rT1, rT2, rT5, and rT6) and were derived from the chromosome harboring the wild-type TSC2 allele (Rose et al. 1999). We concluded that the second hit in these tumors involved deletion of the complete wild-type

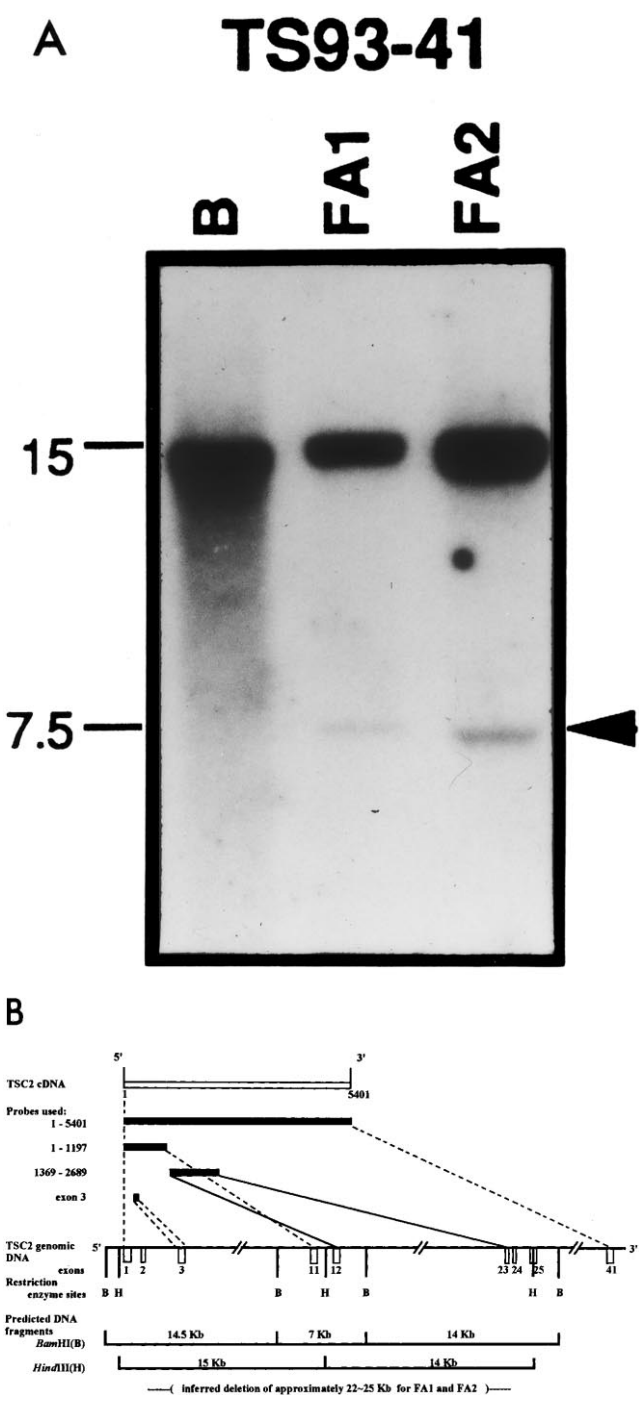

Figure 2 An intragenic deletion of TSC2 is the second (somatic) mutation in two FAs from patient TS93-41. Studies using markers within and flanking TSC2 showed no evidence for LOH in the three FAs obtained. *A*, Southern analysis of DNA from two FAs (FA1 and FA2), performed by means of a TSC2 cDNA probe spanning nucleotide 1–1197, revealed junction fragments of ~7.5 kb with *Hin*dIII digestion. No junction fragment was detected with the lymphocyte DNA (lane B), even when a much higher (approximately fivefold) concentration of DNA was used. *B*, Diagram illustrating probes tested, restriction map for *Bam*HI and *Hin*dIII of the TSC2 gene, predicted fragment sizes, and approximate size and location of deletion.

TSC2 allele, leaving the nonfunctional germline mutated copy in the tumor. Extent of loss along the chromosome was not determined. In the two AMLs from the right kidney that did not show LOH, Southern blotting with the full-length TSC2 cDNA used as a probe did not reveal aberrant banding patterns (data not shown), implying that large deletion or rearrangement of the wild-type TSC2 allele is not the second hit in these two tumors (rT3 and rT4, from patient HOU23-01).

All 14 AMLs of patient TS94-104 showed loss of the normal copy of exon 18 while retaining the 1-bp smaller mutant copy. The markers KG8 and D16S665 also exhibited LOH in DNA from these tumors (table 1). The somatic events led to a minimal deletion, from exons 18–41 of the patient's wild-type TSC2 allele, in these AMLs. Markers TSC2 exon 40 and D16S525 were not informative for this patient (table 1). Because of the lack of informativeness of markers, we were unable to determine whether the LOH differed among samples—that is, whether they represented one very large (clonal) tumor or multiple tumors.

The three FAs obtained from patient TS93-41 showed no decreased intensity for any of the markers tested; however, junction fragments were detected by Southern blotting. DNA extracted from both FA1 and FA2 of patient TS93-41 revealed 7.5-kb junction fragments detectable by restriction enzyme *Hin*dIII (fig. 2*A*). Junction fragments of ~11 kb were detected after digestion with *Bam*HI (data not shown). Three additional probes—nucleotides 1–1197 (exons 1–11), nucleotides 1369–2689 (exons 12–23), and a probe containing only exon 3—were tested to define the deletion (data not shown). On the basis of the published restriction map of the TSC2 genomic DNA (The European Chromosome 16 Tuberous Sclerosis Consortium 1993), the missing portion of the gene is roughly 22–25 kb in size and extends from just beyond exon 3 to exon 25 (fig. 2*B*).

Our study confirmed the germline and defined the somatic second hits of the TSC2 gene in AMLs and FAs from three independent patients with TSC. These observations provide direct proof of tumor-suppressor function for the TSC2 gene, in accordance with Knudson's two-hit hypothesis, in the formation of hamartomas in humans. The studies indicate that the second hit in these tumors involved deletion of the complete wild-type TSC2 allele, leaving the nonfunctional germline mutated copy. Another group has recently reported both hits in a malignant islet-cell tumor from a patient with TSC with a germline mutation identified in the TSC2 gene (Verhoef et al. 1999).

An earlier report (Sampson et al. 1997) found a critical role for the PKD1 gene in the etiology of severe renal disease in some patients with TSC. Sampson's patients exhibited a mixed phenotype that included features of TSC and polycystic kidney disease (PKD). Our three

patients (HOU23-01, HOU23-03, and TS94-104) had serious renal disease resulting primarily from angiomyolipomas with only minor cysts present; therefore, their symptoms are not consistent with PKD. The three patients had single-base-pair insertion or deletion mutations, all of which are predicted to cause premature termination of tuberin, approximately midway (exons 18 and 24) through the open reading frame of the TSC2 gene. We demonstrate that intragenic TSC2 mutations, without involvement of the PKD1 gene, can result in a life-threatening renal phenotype in patients with TSC.

Two of the AML DNAs (rT3 and rT4) from HOU23-01 did not reveal an aberrant banding pattern on Southern blots (not shown), and no LOH of the tested markers was evident; therefore, deletion of the entire wild-type TSC2 allele was not the second event leading to formation of these two tumors. More subtle, undefined mutations are likely the cause of the loss of function of the second allele in these tumors.

Previous attempts to search for LOH of markers within and around the TSC2 gene in facial angiofibromas have been unsuccessful (Henske et al. 1996). Our success in detecting junction fragments relied on the relative abundance of DNA we could extract from the two FAs available and on the use of the two smaller TSC2 cDNA probes (1–1197 and 1369–2689) to detect the specific TSC2 genomic fragments in Southern analyses. The relatively small amount of DNA represented by the junction fragments in these FAs suggests that the tumor cells were in low abundance. A slight reduction of one of the heterozygous alleles of these markers would be difficult to detect. Previous failure to demonstrate LOH in TSC brain lesions could reflect the same difficulties observed in FAs. Perhaps tumorigenesis for FAs and brain tumors does not require the extensive LOH seen in AMLs.

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Stratification Analysis of an Osteoarthritis Genome Screen—Suggestive Linkage to Chromosomes 4, 6, and 16

To the Editor:

We have previously carried out a two-stage genomewide linkage screen for osteoarthritis (MIM 165720) susceptibility loci, using an affected-sibling-pair approach (Chapman et al. 1999). In stage 1 of this screen, we tested 272 microsatellite markers in 297 families, each of which contained at least one pair of siblings who had undergone hip-, knee-, or hip and knee–replacement surgery for primary osteoarthritis. Loci that demonstrated evidence for linkage at nominal $P = .05$ were then taken through to stage 2, in which they were tested against a further 184 families. Sixteen markers within nine genomic regions from stage 1 had evidence of linkage, at $P = .05$. When the data for stages 1 and 2 were combined, the P value decreased for 3 of the 16 loci (D2S202, D11S907, and D11S903) and was constant for a 4th (D11S901). We subsequently concentrated our analysis on the chromosome regions to which these markers map. To test these linkages further, we genotyped additional markers and obtained maximum multipoint LOD scores (MLSs) of 1.2 for chromosome 2 and 3.1 for chromosome 11.

Because there is evidence, from epidemiological, twin, and segregation studies, that the genetic contribution to osteoarthritis differs between the sexes and between different joint groups (Lindberg 1986; Cooper et al. 1994; Kaprio et al. 1996; Chitnavis et al. 1997; Felson et al. 1998), we stratified our chromosomes 2 and 11 linkage data according to sex and site of osteoarthritis (hip or knee). This stratification indicated that the suggestion of linkage to chromosome 2 was principally accounted for by affected sibling pairs with hip osteoarthritis (MLS 2.2), whereas the suggestion of linkage to chromosome 11 was restricted to affected female pairs (MLS 2.8). Because this analysis highlighted substantial differences between the strata tested, we have now reanalyzed stage 1 of our genome screen, for the remaining 20 autosomes, to determine whether any regions harbor susceptibility loci that are obscured in the unstratified data set. We

stratified our stage 1 data into the same six strata tested in our analysis of chromosomes 2 and 11: affected females only (132 families), affected males only (60 families), hips only (194 families), knees only (34 families), female hip (85 families), and male hip (44 families). (A more detailed breakdown of these families can be found in the study by Chapman et al. [1999].) We did not stratify for female knee or male knee, because the number of families was too small (16 and 4, respectively) to allow reliable inference of linkage. Multipoint linkage analysis was performed on the stratified data by means of the ASPEX program.

Ten of the 20 autosomes have one or more multipoint peaks with uncorrected MLS ≥ 1.0 for one or more of the six strata tested (table 1). The highest MLS is 3.9, for chromosome 4q in the female-hip strata, followed by 2.9, for chromosome 6 in the hip-only strata, and 2.1, for chromosome 16 in the female-hip strata. When we adjust MLS values to correct for the seven models tested (one unstratified analysis and six stratified analyses), by deducting $\log_7 = 0.8$ from the original values (Kidd and Ott 1984), chromosome 4 has an MLS value of 3.1, chromosome 6 has an MLS value of 2.1, and chromosome 16 has an MLS value of 1.3. The uncorrected multipoint plots of these three chromosomes are shown in figure 1.

The suggestion of linkage on chromosome 4 is centered on 4q12–4q21.2 and is restricted to female pairs with hip disease. Roby et al. (1999) have recently reported linkage of chromosome 4q to severe early-onset hip osteoarthritis in a large pedigree of Dutch origin. This locus maps to the telomeric end of 4q (4q35), placing it >50 cM distal to the linkage that we have observed. It is therefore unlikely that the two linkages have detected the same locus.

More than 50 cM of chromosome 6 has an uncorrected MLS ≥ 2.0 in the hip-only stratum, between markers D6S257 and D6S262. This region of chromosome 6 contains a strong candidate gene for osteoarthritis, COL9A1 (6q12–6q13). This gene maps within

Table 1

Stratified MLSs

Chromosome	MLS (Corrected)[a]	Cytogenetic Position	Flanking Markers	Stratum
1	1.3 (<1.0)	1q31–1q44	D1S238, D1S103	Female only
3	1.5 (<1.0)	3p25–3p21	D3S1263, D3S1289	Female hip
3	1.5 (<1.0)	3p25–3p21	D3S1263, D3S1289	Female only
3	1.0 (<1.0)	3p21–3p14	D3S1289, D3S1285	Hip only
4	3.9 (3.1)	4q12–4q21.2	D4S398, D4S250	Female hip
4	1.7 (<1.0)	4q12–4q21.2	D4S398, D4S250	Female only
5	1.3 (<1.0)	5p13.3–5q11.1	D5S419, D5S407	Female only
6	2.9 (2.1)	6p21.1–6q22.1	D6S1610, D6S314	Hip only
6	1.8 (1.0)	6p23–6p21.3	D6S422, D6S265	Female hip
6	1.3 (<1.0)	6p21.3–6q15	D6S291, D6S286	Female hip
6	1.2 (<1.0)	6q13–6q22.1	D6S462, D6S314	Male hip
6	1.1 (<1.0)	6p23–6p21.3	D6S422, D6S265	Female only
7	1.5 (<1.0)	7q11.23–7q21.2	D7S502, D7S524	Hip only
7	1.3 (<1.0)	7q22.1–7q32	D7S2502, D7S684	Hip only
8	<1.0 (—)	—	—	—
9	<1.0 (—)	—	—	—
10	<1.0 (—)	—	—	—
12	1.3 (<1.0)	12p12.1–12q11	D12S358, D12S87	Female only
12	1.4 (<1.0)	12q13.3–12q23	D12S43, D12S338	Female hip
12	1.3 (<1.0)	12q13.3–12q23	D12S43, D12S338	Female only
13	<1.0 (—)	—	—	—
14	1.2 (<1.0)	14q24.3–14q32.2	D14S74, D14S51	Male hip
14	1.1 (<1.0)	14q24.3–14q32.2	D14S74, D14S51	Male only
15	<1.0 (—)	—	—	—
16	2.1 (1.3)	16p13.1–16q12.1	D16S407, D16S261	Female hip
16	1.7 (<1.0)	16p13.1–16q12.1	D16S407, D16S261	Female only
16	1.1 (<1.0)	16p13.1–16q12.1	D16S407, D16S261	Hip only
16	2.0 (1.2)	16q21–16q23	D16S265, D16S289	Female only
17	<1.0 (—)	—	—	—
18	1.1 (<1.0)	18p11.32–18p11.1	D18S63, D18S53	Female hip
19	<1.0 (—)	—	—	—
20	<1.0 (—)	—	—	—
21	<1.0 (—)	—	—	—
22	<1.0 (—)	—	—	—

[a] $\log_7 = 0.8$ deducted from the original MLS values.

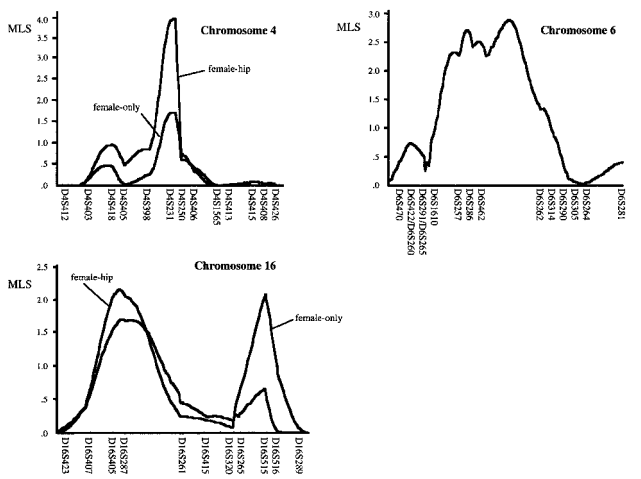

Figure 1 Multipoint analysis. *A*, Chromosome 4, female hip (n = 85 families) and female only (n = 132 families). *B*, Chromosome 6, hip only (n = 194 families). *C*, Chromosome 16, female hip (n = 85 families) and female only (n = 132 families).

the 11-cM interval between D6S257 and D6S286 and encodes the α1 chain of type IX collagen. This collagen is a quantitatively minor cartilage collagen that decorates the type II collagen fibril and that interacts with extrafibrillar macromolecules (Ayad et al. 1994). Two transgenic mouse models have demonstrated that mutations in the equivalent mouse gene can result in an osteoarthritis phenotype. In the first model, a truncated form of the gene resulted in mice with a mild osteochondrodysplasia phenotype and secondary osteoarthritis (Nakata et al. 1993). In the second model, a knockout mouse had no congenital abnormality but developed a severe osteoarthritis that was comparable, in timing and pathology, to human primary osteoarthritis (Fässler et al. 1994). A more detailed analysis of this second model revealed that the synthesis of the α1 polypeptide chain was necessary for type IX collagen assembly (Hagg et al. 1997).

Chromosome 16 does not contain any known genes that can be considered as strong candidates for osteoarthritis susceptibility. As more genes are mapped, candidate loci on this chromosome may become apparent.

Overall, the stratification of our genome screen has revealed additional chromosomal regions that may harbor susceptibility loci for osteoarthritis. Stratification increases the level of genetic homogeneity and can therefore assist in the mapping of loci for complex traits. Our analysis highlights the potential utility of this approach for osteoarthritis.

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Electronic-Database Information

The accession number and URLs for data in this article are as follows:

ASPEX directory, ftp://lahmed.stanford.edu/pub/aspex (for ASPEX software)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for osteoarthritis [MIM 165720])

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Evidence for Interaction between Psoriasis-Susceptibility Loci on Chromosomes 6p21 and 1q21

To the Editor:

Psoriasis (PS [MIM 177900]) is a common inflammatory skin disorder affecting ~2% of the Caucasian population (Nevitt and Hutchinson 1996). The disease is characterized by the occurrence of red scaly patches and, in 5% of cases, is complicated by severe arthritis (Christophers and Sterry 1993). Despite the determining influence of several environmental factors, familial clustering of PS is well established, and empirical recurrence risk in first-degree relatives of isolated patients is in the range of 8%–23% (Hellgren 1967). Moreover, twin studies indicate that PS heritability is in the range of 70%–90% (Farber et al. 1974). An association between PS and the HLA Cw6 antigen has been repeatedly reported (reviewed by Elder et al. 1994), and a susceptibility locus has been identified, by parametric and nonparametric linkage (NPL) analysis (Nair et al. 1997; Trembath et al. 1997; Jenisch et al. 1998; Leder et al. 1998), on chromosome 6p21, within the HLA region. Further candidate loci have been assigned to chromosomes 2q, 4q, 8q, 16q, 17q, and 20p, although confirmation of linkage is so far available only for chromosomes 6p21 and 17q (Tomfohrde et al. 1994; Matthews et al. 1996; Nair et al. 1997; Enlund et al. 1999). Our group has recently identified an additional susceptibility locus in a sample of Italian three-generation psoriatic pedigrees, in which we demonstrated a significant linkage with chromosome 1q21 markers. In particular, NPL scores peaked within the region of the epidermal differentiation complex, between markers D1S1664 (NPL score 4.04) and D1S305 (NPL score 4.07) (Capon et al. 1999). These findings are in agreement with preliminary data obtained in an independent genome scan carried out on 23 U.S. extended pedigrees (Bhalerao and Bowcock 1998).

Since PS is considered a polygenic disorder (Elder et al. 1994), we tested the hypothesis that the 1q21 locus may interact with the one mapping within the HLA region. For this purpose, 15 three-generation families in which PS segregates with chromosome 1q21 markers (i.e., 15 pedigrees displaying positive LOD scores and posterior probabilities of linkage that were >0.6) were selected from our original sample, to investigate the relationship between 1q21 and 6p21 loci. We first tested these 15 families for association with Cw6, by typing one randomly selected trio from each 1q-linked pedigree. In a second phase, we extended HLA-C typing to all family members, and we investigated the correlation between NPL scores at HLA-C and D1S305 loci. Finally, we incorporated evidence of linkage to chromosome 1q21, in assessing linkage at the HLA-C locus.

Analysis 1: testing for association with Cw6.—The protocol described by Tatari et al. (1995) was used for HLA-C molecular typing of the 15 trios, including a randomly selected affected individual and his parents. Association with Cw6 was assessed by means of the transmission/disequilibrium test (TDT [Spielman et al. 1993]), which revealed the presence of the Cw6 antigen in 11 transmitted and 2 nontransmitted chromosomes. One parent was found to be homozygous for Cw6. The *P* value generated by the TDT test was .014, indicating an association with Cw6 in our sample of 1q-linked pedigrees.

Analysis 2: investigating the correlation between NPL scores.—In this phase, HLA-C typing was extended to all family members from the 15 1q-linked pedigrees. The corresponding NPL scores were assessed by use of GENEHUNTER+ 1.3 (Kong and Cox 1997), setting the "single on" and "skip large off" options. As expected, positive NPL scores resulted from the segregation of the HLA-Cw6 allele only (data not shown, available on request).

The correlation between NPL scores at HLA-C and D1S305 loci was investigated by means of SigmaStat 1.0 software (Jandel Scientific), to run Pearson's test. A correlation coefficient of .83 ($P = .0054$) was thus obtained.

Analysis 3: incorporation of evidence of linkage to chromosome 1q21 in assessment of linkage at the HLA-C locus.—This analysis was implemented by use of the ASM 1.0 program (Kong and Cox 1997) exponential model and by application of the proportional weighting scheme described by Cox et al. (1999). In brief, each family was assigned a weight corresponding to its NPL

score for marker D1S305 on chromosome 1q21. A "weighted" LOD score was thus obtained, and the significance of its increment with respect to the HLA-C baseline LOD (i.e., the LOD score calculated without consideration of linkage to 1q21) was assessed by means of a χ^2 test with 1 df (see Cox et al. 1999).

This analysis yielded a "weighted" LOD score of 4.66, whereas the baseline LOD was 2.89. The significance associated with the increased LOD corresponded to a χ^2 of 8.14 ($P = .0043$). This latter P value is comparable to the one generated by the correlation test.

The purpose of this study was to investigate the relationships between HLA-C and 1q21 loci, with respect to their contribution to PS susceptibility. At first, we demonstrated an association with Cw6 in our sample of 1q-linked pedigrees, by means of the TDT test (analysis 1).

The association between PS and HLA-Cw6 has been reproduced in numerous case-control studies performed in different populations (reviewed in Elder et al. 1994). However, it is noteworthy that Tomfohrde et al. (1994) failed to detect association with Cw6 in their sample of three-generation pedigrees that had linkage to chromosome 17q. Barnes et al. (1998) also reported a sample of 115 nuclear families stratified according to the presence of Cw6 among the affected individuals and were able to observe evidence for linkage on chromosome 1q only when analyzing the Cw6 negative sample. Therefore, our data provide the first evidence of association with Cw6 within a sample of families linked to a non-MHC locus. The discrepancy between our results and those reported by Barnes et al. (1998) might be due to the existence of two distinct susceptibility loci lying close to each other on chromosome 1q21, each interacting with different gene products. Since the 1q21 region contains >30 genes regulating epidermal growth and differentiation (Marenholz et al. 1996), this hypothesis is not altogether unlikely. On the other hand, the discrepancy between the two sets of results might be accounted for by differences in methods, population, and/or sample composition.

In the second phase of this study (analysis 2), the 15 1q-linked families were subjected to an analysis of the correlation between the NPL scores at HLA-C and D1S305 loci. In fact, the use of correlation between LOD scores has long been suggested as a means to assess interaction between unlinked regions (MacLean et al. 1993). This method has recently been extended to the analysis of complex disorders and NPL scores, by Cox et al. (1999). The correlation coefficient of .83 ($P = .0054$) that we report here was computed on a sample selected for an allele-sharing excess at 1q21 markers. NPL score and correlation analysis show that these families also tend to display an allele-sharing excess at the HLA-C locus. Thus, our data can be interpreted as preliminary evidence of an epistatic interaction between the 1q21 and 6p21 PS-susceptibility loci.

In the last phase of this study (analysis 3), assuming an interaction between 6p21 and 1q21 loci, we computed a "weighted" NPL score at the HLA-C locus. Thus, we could observe a significant increment of the "weighted" LOD score (4.66) with respect to the baseline LOD (2.89). This provided the first significant evidence for linkage, in the Italian population, with the HLA region. In fact, in a previous study, we had failed to detect any significant LOD score between marker D6S273 (mapping close to HLA-C) and PS (Capon et al. 1999); moreover, the Cw6 baseline LOD that we have reported in this study is on the threshold of statistical significance. Thus, only the assumption of interaction allowed us to replicate the linkage to the HLA region. This suggests that some of the difficulties in replication of results obtained in genome scans for PS susceptibility and, more generally, for complex disorders might be smoothed in the near future, by analyses allowing identification of potential interactions.

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Founder *BRCA1/2* Mutations among Male Patients with Breast Cancer in Israel

To the Editor:

Germline mutations in the *BRCA1* (MIM 113705) and *BRCA2* (MIM 600185) genes are associated with a high risk of breast and ovarian cancer in women (Ford et al. 1998). The risk of male breast cancer is higher among carriers of *BRCA2* mutations compared with carriers of *BRCA1* mutations, although the absolute risk to male carriers is not well characterized (Wooster et al. 1995; Ford et al. 1998). Studies of *BRCA2* in small population- and clinic-based series of male breast cancer patients from the United States and the United Kingdom have found carrier frequencies of 4%–14% (Couch et al. 1996; Friedman et al. 1997; Mavraki et al. 1997). Carrier frequencies for specific founder *BRCA2* germline mutations have been higher: 21%–40% (Thorlacius et al. 1996; Haraldsson et al. 1998; Csokay et al. 1999). The number of cases analyzed has ranged from 18 to 54, however, and the confidence limits on the carrier frequencies are very large. Among predominantly Ashkenazi Jewish populations, three founder mutations in the *BRCA1/2* genes are present in >2% of all individuals, and they may account for ~80% of all the mutations in these genes (Struewing et al. 1997; Frank et al. 1998). This allows the relatively efficient analysis of larger numbers of cases.

All male patients with breast cancer ($n = 165$) diagnosed in five Israeli hospitals during 1980–97 were reviewed for inclusion in this study. Jewish patients were characterized as Ashkenazi or non-Ashkenazi, on the basis of either the recorded place of birth in the Israeli Population Registry or, if they were born in Israel, their father's recorded place of birth. Non-Ashkenazi Jews were born in Turkey, Syria, Iraq, Iran, Afghanistan, Morocco, or Libya. Israeli-born non-Jews were designated as Arab, Christian, or Druze. Samples were analyzed anonymously. Among the 122 histologically verified cases for whom adequate pathological material could be obtained, 1 was excluded, because analysis from multiple specimens differed with respect to mutation status and marker genotypes. Eighty-nine cases were Ashkenazi, 21 were non-Ashkenazi, and 14 were Arabs.

Unstained 5-μm paraffin sections were scraped from slides and were digested in 100 μl of buffer containing 10 mM Tris (pH 8.0), 50 mM KCl, 10 mM EDTA, 2 mM $MgCl_2$, 1% Tween-20 and 10 μg of Proteinase K at 57°C overnight. The samples were then heated to 100°C for 5 min and then 1–5 μl were used as template in the PCR reactions. A 20-μl multiplex PCR reaction was used to amplify the three segments containing the founder mutations. Each reaction contained 1 × PCR Buffer, 2.5 mM $MgCl_2$, 100 μM each dNTP, 15% sucrose, and 0.75 U *Taq* Gold (PE Biosystems). Primers included 300 nM 2F103 (6FAM-tcgcgttgaagaagtacaaaatgtc), 300 nM 2R102 (caaattaatacactcttgtgctgacttac), 200 nM 20F101 (HEX-gtcaatggaagaaaccaccaaggtc), 200 nM 20R101 (tgcaaaggggagtggaatacacagt), 200 nM 11F101 (TET-tagggaagcttcataagtcagtctca), and 200 nM 11R101 (cttgcgttttgtaatgaagcatct). Cycling consisted of 94°C for 12 min, followed by 10 cycles of 92°C for 10 s, annealing at 68°C for 10 s, and 72°C for 20 s, with the annealing temperature being decreased 1.5°C per cycle, followed by 30 cycles of 92°C for 15 s, 55°C for 15 s, and 72°C for 30 s, followed by 72°C for 10 min. The PCR products were diluted 1:149 with water and then were diluted 1:6 with loading cocktail, according to the manufacturers' recommendations, and were electrophoresed on an ABI 310 capillary electrophoresis machine by use of GENESCAN software (PE Biosystems). Known mutant, wild-type, and no-DNA controls were run with each PCR reaction. Mutants were identified by visual inspection of the electropherograms, with the small-insertion or -deletion mutations resulting in an extra peak in the pattern. Primers to amplify the chromosome 13 markers D13S260, D13S1695, D13S1698, and D13S1701 were redesigned to result in shorter amplicons. All carriers of the 6174delT mutation and a reference family with this mutation were analyzed for these four markers, on the ABI 310 (primer sequences are available, on request, from the corresponding author.)

The results of mutation testing are shown in table 1. Of the 19 mutation carriers, 17 were Ashkenazi and 2 were non-Ashkenazi Jews. Carrier frequencies were not statistically different between the two ethnic groups ($P = .7$). None of the Arab men was a carrier, and the 5382insC mutation was not detected in any patients. The mean ages at diagnosis of all 19 mutation carriers (64 years) and of the *BRCA2* 6174delT mutation carriers (66 years) were not significantly different than that of noncarriers (68 years). Reliable genotypes could not be obtained for all the markers for all carriers, but 14 of 14 carriers successfully genotyped at D13S1698 shared the same allele as was seen in the reference family; 12 of 12 carriers successfully genotyped at D13S260 shared the correct allele; 7 of 8 carriers successfully genotyped at D13S1701 shared the correct allele; and 13 of 15 carriers successfully genotyped at D13S1695 shared the correct allele.

These results suggest that ⩾17% of Jewish men diagnosed with breast cancer in Israel carry a mutation in *BRCA1* or *BRCA2*, since only the three common founder mutations were screened. This study was based on the analysis of a small amount of pathological tissue, and the proportion of the tissue that was tumor was unknown. Our methods may have led both to false negatives, caused by the complete loss of the relevant genes in a tumor—although this might be expected to result in preferential loss of the wild-type and retention of the mutant allele—and false positives, caused by contamination of the specimens during processing and handling. Our observed carrier frequency is somewhat higher than those in most series studied in the United States and the United Kingdom but is lower than frequencies observed in several other populations with common founder mutations. Unlike that in female carriers, who have an earlier age at diagnosis of breast cancer, the age at diagnosis among male carriers was not significantly lower than among noncarriers. This may reflect the fact that most men carried a *BRCA2* mutation, because the age at diagnosis for female breast cancer appears to be later for *BRCA2* carriers than for *BRCA1* carriers (Ford et al. 1998).

The *BRCA1* 185delAG founder mutation has been observed in both Ashkenazi and non-Ashkenazi Jewish women with breast or ovarian cancer (Bar-Sade et al.

Table 1

Israeli Male-Breast-Cancer Mutation Testing Results

Population	No. Tested	No. (%) Positive for Mutation [95% CLs]			
		BRCA1		*BRCA2*	
		185delAG	5382insC	6174delT	Total
Jews:					
All	110	4 (3.6) [1, 9]	0	15 (13.6) [8, 21]	19 (17.3) [11, 26]
Ashkenazi	89	4 (4.5) [1, 11]	0	13 (14.6) [8, 24]	17 (19.1) [12, 29]
Sephardic	21	0	0	2 (9.5) [1, 30]	2 (9.5) [1, 30]
Arabs	14	0	0	0	0

1998). We observed the 6174delT mutation in two non-Ashkenazi Jewish men with breast cancer, and this is the first published observation of this mutation in this population (Neuhausen et al. 1998). Our characterization of Jewish men as Ashkenazi or non-Ashkenazi was based on the place of birth as recorded in the Israeli Population Registry. Since ethnic origin was not determined in person or by biological testing, some misclassification could have occurred. If verified in other studies, however, this finding would suggest that this mutation is considerably older than previously estimated (Neuhausen et al. 1998). Genotyping of four microsatellite markers near *BRCA2* was consistent with their sharing the same haplotype as was seen in Ashkenazi carriers of this mutation. Although the number of cases was small, we did not observe an earlier age at onset among mutation carriers, nor did we detect any of the three mutations in Arab men with breast cancer.

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Heterogenous Point Mutations in the Mitochondrial tRNA Ser(UCN) Precursor Coexisting with the A1555G Mutation in Deaf Students from Mongolia

To the Editor:

Several mitochondrial mutations have been identified that exhibit matrilineal transmission of deafness either in isolation or with other features such as diabetes. The A1555G substitution in the 12S rRNA gene is known to be associated with nonsyndromic deafness, with or without aminoglycoside exposure, in several different populations (Higashi 1989; Hu et al. 1991; Jaber et al. 1992; Prezant et al. 1993; Pandya et al. 1997; Estivill et al. 1998). Hamaski et al. (1997) demonstrated specific binding of aminoglycoside antibiotics to the proposed 1555A→G transition in a mitochondrial 12S rRNA construct, providing evidence in support of the aminoglycoside-ototoxicity hypothesis suggested by Prezant et al. (1993). However, the explanation for tissue specificity and for the pathogenesis in individuals without exposure to aminoglycosides remains unclear. An additional mutation in a nuclear gene has been postulated as one possible cause for the latter, but efforts to identify such a modifier gene have not been successful (Guan et al. 1996; Bykhovskaya et al. 1998). Another mitochondrial mutation, A7445G, was initially identified, in a Scottish pedigree, as a cause of sensorineural hearing loss with incomplete penetrance (Reid et al. 1994; Vernham et al. 1994). In two subsequent pedigrees, from New Zealand and Japan, a relatively mild palmoplantar keratoderma (MIM 148350) was also noted in most affected individuals (Sevior et al. 1998). The A→G substitution at position 7445 changes the final residue of the cytochrome oxidase (COI) stop codon on the H strand and is also located immediately adjacent to the *3′* end of tRNA ser(UCN), whose precursor is encoded on the L strand. The A7445G mutation was recently shown to retard the normal processing of the tRNA precursor, resulting in a 70% reduction in tRNA ser(UCN) production and in a 45% decrease in protein synthesis (Guan et al. 1998). A simultaneous decrease in mRNA for the NADH (nicotinamide adenine dinucleotide [reduced]) dehydrogenase (complex I) ND6 subunit may reflect an upstream effect of the delayed processing of the polycistronic RNA2 molecule. One or more of these three defects could contribute to the pathogenesis of deafness (Guan et al. 1998). In previous work we documented the A1555G mutation in three Mongolian families with matrilineal deafness (Pandya et al. 1997). To estimate the prevalence of both these mitochondrial mutations in the deaf population in Mongolia, we screened 480 deaf students and family members ascertained through the only residential School for Deaf and Blind in Mongolia, which is located in Ulaanbaatar, as well as 389 Mongolian controls with normal hearing. Our sample is estimated to include >70% of the student-age deaf population of Mongolia. We identified 37 deaf students with the A1555G mutation and 9 with the 7445 mutation, by screening for loss of a restriction site for the *Alw*26I and the *Xba*I restriction enzymes, respectively. Direct sequencing of DNA from the nine individuals positive for the loss of an *Xba*I restriction site revealed heterogeneity with novel mutations at one of three adjacent base pairs—7443, 7444, or 7445. Six students had coexistent A1555G and G7444A substitutions, which has not previously been reported.

After informed consent was provided, the clinical history was obtained and an examination was performed on each subject by one of the investigators, with special emphasis on identification of environmental causes of deafness, such as trauma, meningitis, otitis media, or pharmacologic ototoxicity, as well as syndromic forms of genetic deafness. An audiogram was obtained on most deaf individuals, with air conduction being tested at 250, 500, 1,000, 2,000, 3,000, 4,000, 6,000, and 8,000 Hz. For initial screening, DNA samples were tested for the presence of A1555G and A7445G mitochondrial mutations, by PCR amplification and subsequent digestion with *Alw*26I and *Xba*I restriction enzymes, as described elsewhere (Prezant et al. 1993; Reid et al. 1994; Pandya et al. 1997). The primer pairs used for PCR amplification were as follows: for the 1555 region, nucleotide (nt) 1261–1282 of the Cambridge sequence (Anderson et al. 1981) was used for the forward primer, and nt 2866–2843 was used for the reverse primer. For the 7445 region, nt 7178–7198 and nt 7840–7821 were used for the forward and reverse primers, respectively. The amplification products were resolved on a 2% NuSieve gel, were stained with ethidium bromide, and were photographed. On the basis of the restriction analysis, 31 individuals were found to be positive for the A1555G mutation alone, 3 were positive for the 7445 mutation alone, and 6 were positive for both (fig. 1).

To confirm the nature of the mutations, sequencing of DNA samples was performed by means of the Sequenase PCR-product sequencing kit from Amersham Life Science. Approximately 200 ng of the PCR product was incubated with 10 U of exonuclease 1 and 2 U of alkaline phosphatase, at 37°C for 30 min (Hanke and Wink 1994), followed by heat inactivation at 80°C for 15 min. The appropriate forward or reverse primer was added to an aliquot of the enzyme-treated PCR product, followed by the labeling solution mix made up of 40 mM Tris-Hcl (pH 7.5), 20 mM $MgCl_2$, 50 mM NaCl, 5 mM DTT, 0.375 μM 7-deaza dNTP = s, 10 μCi [^{35}S-α] dATP, and 3.2 U Sequenase. A 4-μl aliquot of this mixture was transferred to a tube containing 2.5 μl of

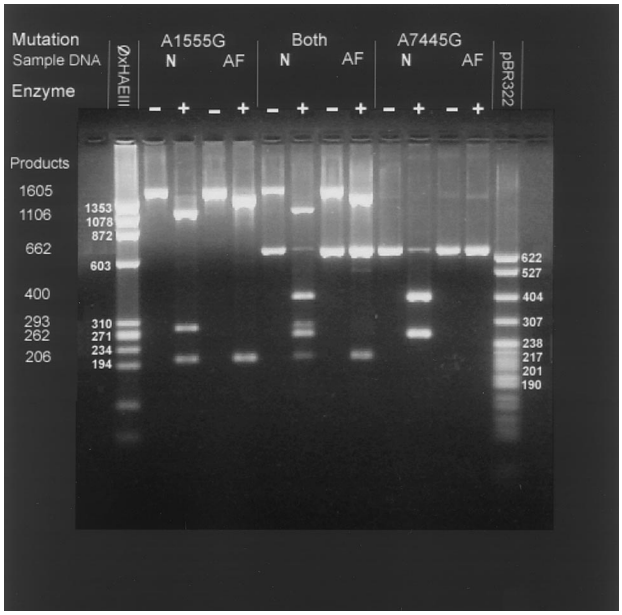

Figure 1 Representative gel with restriction enzyme digestion pattern of individuals with the A1555G mutation alone (lanes 4 and 5), with the 7445 mutation alone (lanes 12 and 13), and with both mutations (lanes 8 and 9). The band pattern for individuals with normal hearing is depicted for each category with and without digestion with the appropriate restriction enzyme. The A1555G mutation abolishes a site for the *Alw*26I enzyme. The undigested PCR product of 1605 bp is digested in normal individuals to yield bands of 1106, 293, and 206 bp. In affected individuals, lack of digestion results in a larger band at 1399 bp and in the lower band at 206 bp. The PCR product obtained with primers for the nt7445 mutation is 662 bp. In unaffected individuals, digestion with the restriction enzyme *Xba*I results in two bands—400 and 262 bp in size. The presence of a mutation at this nt abolishes the recognition sequence for this enzyme with absence of digestion after incubation with the enzyme.

the ddNTPs and incubated at 37°C for 10 min. The reaction was stopped with 4 μl of stop dye, and the samples were denatured and immediately loaded onto a 6% 1 × GTG acrylamide sequencing gel. After electrophoresis, the gel was dried and placed on an x-ray plate overnight, before the film was developed. Primers used to sequence the 1555 region were as described elsewhere (Pandya et al. 1997). The sequencing primers for the 7445 region were as follows: forward (nt 7321–7341) and reverse (nt 7623–7600). Ten of the 31 samples that were positive for the loss of a restriction site at 1555 were sequenced, and all had the A1555G substitution, as expected (data not shown). In contrast, direct sequencing of DNA from the nine individuals with loss of the *Xba*I restriction site showed mutational heterogeneity, as seen in figure 2. Two of the three individuals with the isolated loss of the *Xba*I restriction site had an A7445C substitution, whereas the third had an A7443G substitution. All six students with the double mutation showed a G7444A substitution. This mutation has been reported elsewhere in association, with Leber hereditary optic neuropathy (LHON) in whites, but it has been considered to be a secondary change that may increase the penetrance of the primary mutation but that is insufficient in itself to cause LHON (Brown et al. 1995). The G7444A substitution has not been reported in association with deafness, however. All the mutations appeared homoplasmic in every affected individual who tested positive, within the limits of detection by PCR-RFLP analysis and direct sequencing of PCR products. No subject with loss of either restriction site was detected among the 389 control samples from hearing Mongolians. Overall, our results indicate an incidence of 7.7%, 0.33%, 0.33%, and 1.33% for the A1555G, A7443G, A7445C, and A1555G + A7444G changes, respectively, in our population of deaf students.

The six individuals with the G7444A + A1555G double mutation included five with matrilineal transmission of hearing loss and one with no family history of deafness. A definite history of aminoglycoside exposure could be elicited in only two individuals, but all six had severe to profound bilateral sensorineural hearing loss detected at birth or during early infancy. Both individuals with the isolated A7445C mutation had onset of deafness after an illness at age 1–4 years, and the one individual with the A7443G mutation had been deaf since infancy. These findings differ from those reported for other families with the A7445G mutation, in that the Scottish family exhibited incomplete penetrance (Reid et al. 1994) and the New Zealand family had a late onset (in the second decade) of hearing loss, with subsequent progression to profound deafness and palmoplantar keratoderma (Sevior et al. 1998). The variability in phenotypic expression noted in these reports was attributed to differences in the background mitochondrial haplotypes of the two pedigrees, thereby implying an interaction between the A7445G mutation and a second mitochondrial change. Although a history of aminoglycoside use was present in two of the six individuals, it did not appear to be a prerequisite, in view of the fact that some affected individuals had hearing loss detected soon after birth. The phenotype of deafness in individuals with isolated A1555G mutation is quite variable, with some reported patients developing late-onset hearing loss in the absence of aminoglycoside exposure (Estivill et al. 1998). These observations raise the possibility that the coexistence of the two mutations may influence the age at onset and severity of hearing loss, even in the absence of aminoglycoside exposure. The sample size is too small to make any definitive conclusions, and further observations would be required to establish digenic epistasis as a likely explanation for the apparent phenotypic heterogeneity in these families.

The A7445G mutation is located in the final residue of the COI gene on the H strand. It alters the normal

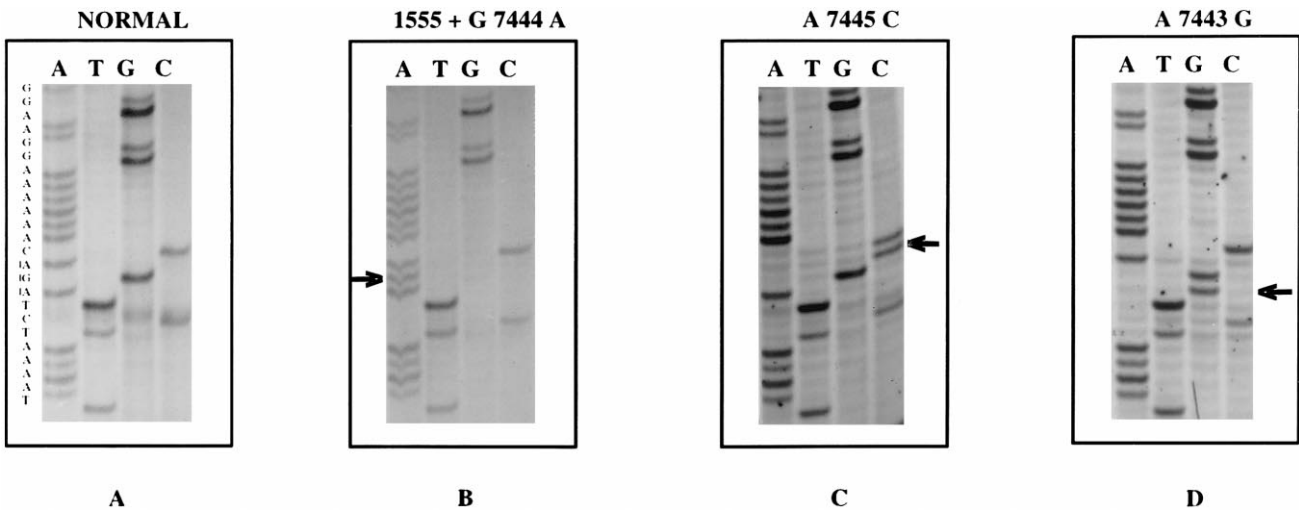

Figure 2 Sense strand sequence of the 3′ region of the tRNA ser(UCN) in a normal individual (*A*) and in three deaf students (*B-D*) from Mongolia. *B*, A deaf student with the double mutation showing the AGA→AAA substitution at nt7444. *C*, A deaf student with isolated nt 7445 mutation, with substitution of AGA→AGC. *D*, A deaf student with isolated nt 7443 change, with AGA→GGA substitution.

stop codon, AGA, to another stop codon, AGG, and should therefore be a silent change. However, this substitution has been shown to dramatically alter the processing of the tRNA ser(UCN) precursor on the L strand, as noted previously. It also inhibits the maturation of other, more distal genes on the L strand, by destabilizing the major mitochondrial polycistronic transcription product, RNA 2 (Guan et al. 1998). All three newly described substitutions—A7445C, G7444A, and A7443G—also abolish the *Xba*I cleavage site at nt7445. Since all are also associated with deafness, it seems plausible that they may share with A7445G a common pathogenic mechanism, possibly involving interference with the activity of the endogenous 3′ nuclease, which is required for normal processing of the tRNA ser(UCN) precursor. If so, we would predict that these three mutations would act similarly to A7445G and would alter tRNA ser(UCN) and upstream NADH dehydrogenase (complex I) ND6 subunit–message maturation, as well as protein synthesis. An RNASE P–like enzyme and a tRNA 3′ endonuclease have been implicated as the key enzymes in the precise cleavage at the 5′ and 3′ ends of mitochondrial precursor tRNAs (Rossmanith et al. 1995). Although these enzymes have been isolated and partially purified (Rossmanith 1997; Rossmanith and Karwan 1998), their number, molecular structure, and substrate recognition sequences have not yet been well characterized. On the H strand, however, the three new mutations we have described would all result in a readthrough of the normal stop codon of the COI message, leading to the addition of three amino acids to the protein. The predicted tripeptides additions are SerGlnLys, LysGlnLys, and ArgGlnLys for the A7445C, G7444A, and A7443G substitutions, respectively. These changes are not lethal but could certainly contribute to the observed phenotype by altering the activity of COI either directly or by modifying the assembly of cytochrome oxidase (complex IV) holoenzyme. The resulting deficit in mitochondrial energy transduction may in turn contribute to defective development and function of the auditory sensory tissues. Hence, the potential involvement of the identified mutations in the synthesis and integrity of COI and in mitochondrial RNA processing provide two likely targets for further analysis of the molecular basis of the phenotype of deafness.

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