§Haematology and Oncology, Royal Children's Hospital, Parkville, Victoria, Australia

¶Department of Radiology, Royal Children's Hospital, Parkville, Victoria, Australia

Correspondence to: Dr Halliday, halliday@cryptic.rch.unimelb.edu.au

- Bertina RM, Koelman BP, Koster T, Rosendaal FR, Dirven RJ, Ronde Hd. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994;**369**:64-7.
- Zoller B, Dahlback B. Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis. *Lancet* 1994;**343**:1536-8.
- Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterised by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci USA* 1993;**90**:1004-8.
- Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 1996;**88**:3698-703.
- Metz J, Kloss M, O'Malley CJ, Rockman SP, De Rosa L, Doig RG, McGrath KM. Prevalence of factor V Leiden is not increased in women with recurrent miscarriage. *Clin Appl Thromb/Hemo* 1997;**3**:137-40.
- Pecheniuk NM, Marsh NA, Walsh TP, Dale JL. Use of first nucleotide change technology to determine the frequency of factor V Leiden in a population of Australian blood donors. *Blood Coag Fibrin* 1997;**8**:491-5.
- Nowak-Gottl U, Strater R, Dubbers A, Oleszuk-Raschke K, Vielhaber H. Ischaemic stroke in infancy and childhood: role of the Arg506 to Gln mutation in the factor V gene. *Blood Coag Fibrin* 1996;**7**:684-8.
- Ganesan V, Kelsey H, Cookson J, Osborn A, Kirkham FJ. Activated protein C resistance in childhood stroke. *Lancet* 1996;**347**:260.
- Zenz W, Bodo Z, Plotho J, Streif W, Male Ch, Bernert G, Rauter L, Ebetsberger G, Kaltenbrunner K, Kurnik P, Lischka A, Paky F, Ploier R, Hofler G, Mannhalter C, Muntean W. Factor V Leiden and prothrombin gene G 20210 a variant in children with ischemic stroke. *Thromb Haemostas* 1998;**80**:763-6.
- Aschka I, Aumann V, Bergmann F, Budde E, Eberl W, Eckhof-Donovan S, Krey S, Nowak-Gottl U, Schobess R, Sutor AH, Wendisch J, Schneppenheim R. Prevalence of factor V Leiden in children with thrombo-embolism. *Eur J Pediatr* 1996;**155**:1009-14.
- Vielhaber H, Ehrenforth S, Koch H, Scharrer I, Werf Nvd, Nowak-Gottl U. Cerebral venous sinus thrombosis in infancy and childhood: role of genetic and acquired risk factors of thrombophilia. *Eur J Pediatr* 1998;**157**:555-60.
- McColl MD, Chalmers EA, Thomas A, Sproul A, Healey C, Rafferty I, McWilliam R, Eunson P. Factor V Leiden, prothrombin 20210 G→A and the MTHFR C677T mutations in childhood stroke. *Thromb Haemostas* 1999;**81**:690-4.
- Thorarensen O, Ryan S, Hunter J, Younkun DP. Factor V Leiden mutation: an unrecognized cause of hemiplegic cerebral palsy, neonatal stroke and placental thrombosis. *Ann Neurol* 1997;**42**:372-5.
- Rai R, Regan L, Hadley E, Dave M, Cohen H. Second-trimester pregnancy loss with activated protein C resistance. *Br J Haematol* 1996;**92**:489-90.
- Grandone E, Margaglione M, Colaizzo D, d'Addetta M, Cappucci G, Vecchione G, Scianname N, Pavone G, Di Minno G. Factor V Leiden is associated with repeated and recurrent unexplained fetal losses. *Thromb Haemostas* 1997;**77**:822-4.
- Blair E, Stanley F. Intrapartum asphyxia: a rare cause of cerebral palsy. *J Pediatr* 1988;**112**:515-19.
- Nacye RL, Peters EC, Bartholomew M, Landis JR. Origins of cerebral palsy. *Am J Dis Child* 1989;**143**:1154-61.
- Yudkin PL, Johnson A, Clover LM, Murphy KW. Assessing the contribution of birth asphyxia to cerebral palsy in term singletons. *Paediatr Perinat Epidemiol* 1995;**9**:156-70.
- Holm VA. The causes of cerebral palsy. A contemporary perspective. *JAMA* 1982;**247**:1473-7.
- Pharoah P, Cooke T, Rosenbloom L. Acquired cerebral palsy. *Arch Dis Child* 1989;**64**:1013-16.
- Dizon-Townson DS, Nelson LM, Easton K, Ward K. The factor V Leiden mutation may predispose women to severe preeclampsia. *Am J Obstet Gynecol* 1996;**175**:902-5.
- Ros HS, Cnattingius S, Lipworth L. Comparison of risk factors for preeclampsia and gestational hypertension in a population-based cohort study. *Am J Epidemiol* 1998;**147**:1062-70.

J Med Genet 2000;**37**:789-791

Analysis of the human tumour necrosis factor-alpha (TNFα) gene promoter polymorphisms in children with bone cancer

EDITOR—TNFα (tumour necrosis factor-alpha) is a cytokine produced by macrophages and monocytes with a wide range of activities, and polymorphisms within this gene have been postulated to contribute to MHC associations with autoimmune and infectious diseases.¹ The role of TNFα in cancer is a controversial matter, because while it plays a key role in the "in vitro" killing of tumour cells by macrophages and lymphocytes, it has also been found in high concentrations in patients with cancer, suggesting that it may be an endogenous tumour promoter "in vivo".^{2,3} Different results with several tumour types show that TNFα may have both tumour necrotic and tumour promoting activities.

Recently, several genetic polymorphisms have been described in the human TNFα promoter.⁴⁻⁶ Among them, the rare allele at position -308 (TNF308.2) has been proven to be part of a complex haplotype that is involved in higher TNFα levels and has been related to poor prognosis in several diseases.⁷ The existence of different TNFα alleles, related to different levels of TNFα, raises the possibility that tumour development is somewhat related to the genetic propensity of the person to produce higher levels of TNFα and, therefore, with the presence of genetic variants in this gene. In fact, an increase in the TNF308.1/TNF308.2 genotype has been reported in different tumour types, with a significantly increased frequency of the TNF308.2 allele in patients with malignant tumours.⁸ Wilson *et al*⁹ have shown that the polymorphism at -308 has a significant effect on the transcriptional activity of the human TNFα gene, either because the interaction of the transcription enhancers is increased owing to the different

DNA conformations, or because the TNF2 variant is the target for novel binding proteins.

The G to A transition at position -238 (TNF238.2) is also suspected to influence the expression of TNFα, and although its clinical and functional consequences are not clear so far,⁹ it has been associated with development and prognosis of different diseases as well.^{10,11}

The effects of TNFα on osteoblast differentiation and proliferation are complex but it is generally assumed that it inhibits bone formation and stimulates bone resorption.¹² The activity of TNFα in osteosarcoma, Ewing's sarcoma, and primary human osteoblast cultures has been widely studied showing that it has an antiproliferative and cytotoxic role which usually depends on the type of cell line under analysis.^{13,14}

Synergistic cytotoxicity has been described between TNFα and other cytokines, most frequently IFNγ (interferon gamma) and also with certain drugs that are commonly used in the treatment of osteosarcoma and Ewing's sarcoma like the topoisomerase II inhibitors.¹⁵ The data available indicate that TNFα and agents that stimulate its production by host macrophages may have a role in the treatment of osteosarcoma and Ewing's sarcoma.

Based on the role of TNFα in bone biology and the growing evidence of the relationship existing between TNFα genetics and cancer, we tried to test the hypothesis of whether genetic polymorphisms of the TNFα promoter contribute to the pathogenesis or prognosis of paediatric bone tumours.

DNA was extracted following standard procedures¹⁵ from peripheral blood lymphocytes of 110 paediatric patients (52 females, 58 males) with bone tumours (63 osteosarcomas and 47 Ewing's sarcomas) and 111 healthy children (53 females, 58 males). All the subjects included in the analysis were white, most of them from the region of Navarre (Spain), and the age distribution was very similar in the tumour and control groups (mean (SD): osteosarcoma 13.5 years (SD 3.3), Ewing's sarcoma 12 years (SD 3.7), controls 11.1 years (SD 5.1)).

Figure 1 DGGE gel showing polymorphic and normal band patterns. Lanes 1 to 3: genotype normal/-376-238. Lane 4: genotype normal/-308. Lanes 5 and 6: normal, non-polymorphic patterns.

Table 1 Genotypes for the *TNFA* gene promoter in the control subjects and the bone tumour groups

Genotypes	Controls (n=111) *		Osteosarcoma (n=63)		ES† (n=47)	
	No	%	No	%	No	%
Normal/normal	67	60.4	47	74.6	35	74.5
Normal/-308	26	23.4	13	20.6	6	12.8
Normal/-238-376	11	9.9	2	3.2	4	8.5
-308/-238-376	2	1.8	0	0	0	0
Normal/-238	5	4.5	0	0	2	4.3
-308/-308	0	0	1	1.6	0	0

*Number of subjects analysed.

†ES, Ewing's sarcoma.

The human *TNFA* promoter region between nucleotides -398 and -103 was analysed by PCR-DGGE (polymerase chain reaction coupled to denaturing gradient gel electrophoresis) as previously published^{16 17} (fig 1).

Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS, version 9.0). The chi-square contingency test, with Yates's modification for small numbers if needed, was used to test for a significant association between disease and *TNFA* genotypes or haplotypes. Crude odds ratios (ORs) were calculated and given with 95% confidence intervals (CI). To test for association between clinical parameters and genotypes or haplotypes, *t* tests or one way analysis of variance (ANOVA) were performed. Statistical significance was assumed if $p \leq 0.05$ and highly statistical difference if $p < 0.01$; differences between variables with $p > 0.05$ were considered not statistically different. Osteosarcomas and Ewing's sarcomas were treated independently in the statistical analyses given that they are different clinical entities, both in genetic background and in the clinical and histological aspects.

We have analysed the distribution of the *TNFA* gene promoter polymorphisms at positions -376, -308, -238, and -163 in 110 bone sarcoma paediatric patients and

111 paired healthy children, to search for the putative association between the *TNFA* gene and tumour development or prognosis.

The overall distribution of *TNFA* polymorphic alleles in bone cancer paediatric patients and control subjects is shown in table 1. As in previous reports, polymorphisms at -376 and -238 were found to be in linkage disequilibrium.⁵ To date, there are no consistent data on the frequency of the polymorphic alleles at -376 and -163 in white populations, and larger series should be screened to establish the frequencies for these alleles in both normal and disease related populations.

The frequencies of the alleles at -308 and -238 in the Spanish population (0.87 and 0.92, respectively, deduced from table 2) were found to be very similar to those described for European white populations.^{10 18}

Tables 2 and 3 show the allele and genotype frequencies of the genetic variants of the *TNFA* gene promoter at -308 and -238 in both cancer patients and healthy subjects. The frequency of the TNF238.2 allele (adenine at position -238) and TNF238.1/TNF238.2 heterozygote genotype was significantly lower in the osteosarcoma group, but not among Ewing's sarcomas. We did not find any outstanding difference in the distribution of the TNF308.2 allele (adenine at position -308) between bone sarcomas and controls.

No relationship was found between the presence of genotypes for the *TNFA* gene promoter and any of the clinical parameters tested: tumour stage, tumour location or size, development of metastasis or relapse, and age at diagnosis (data not shown).

Surprisingly, the percentage of males carrying polymorphisms of the *TNFA* promoter was statistically higher than in females ($p=0.025$, OR=4.05, CI=1.13-14.43). We did not detect this difference in the group of healthy controls, in which the distribution of polymorphic patterns was similar in both sexes.

Several reports have indicated that different HLA (human leucocyte antigen) products and related genes may be risk factors for and also protective factors against cancer. The *TNFA* gene is of particular interest because of its involvement in tumour immunity and cancer pathogenesis and the relationship existing between certain *TNFA* genetic variants and human tumours.

Although the exact regulatory mechanisms altered by the *TNFA* promoter polymorphisms are not completely delineated, studies on these polymorphisms have shown that those at -308 and -238 are associated with the development and even prognosis of certain types of cancer. Chouchane *et al*⁶ detected a marked decrease of the TNF308.1 homozygous genotype in patients with non-Hodgkin's lymphoma, breast carcinoma, and in a group of different malignant tumours. Nevertheless, it must be taken into account that the results of this study were obtained with

Table 2 Frequencies of the *TNFA* promoter alleles -238 and -308 in the paediatric bone sarcoma groups compared to control children

	Controls (n=222) *		Osteosarcoma (n=126)		ES† (n=94)	
Allele	No	%	No	%	No	%
<i>Locus TNFa -308</i>						
G (TNF308.1)	194	87.4	111	88.1	88	93.6
A (TNF308.2)	28	12.6	15	11.9	6	6.4
			p=0.847, NS, OR=0.94 (CI 0.48, 1.83)		p=0.102, NS, OR=0.47 (CI 0.19, 1.18)	
<i>Locus TNFa -238</i>						
G (TNF238.1)	204	91.9	124	98.4	88	93.6
A (TNF238.2)	18	8.1	2	1.6	6	6.4
			p=0.012, ‡, OR=0.18 (CI 0.042, 0.8)		p=0.6, NS, OR=0.77 (CI 0.29, 2.01)	

*Number of alleles analysed.

†ES, Ewing's sarcoma.

‡Statistically significant.

Table 3 Genotypes for the *TNFA* promoter variants at -238 and -308 in bone sarcoma patients and in healthy controls

Genotype	Controls (n=111) *		Osteosarcoma (n=63)		ES† (n=47)	
	No	%	No	%	No	%
<i>Locus TNFA -308‡</i>						
GG	83	74.8	49	77.8	41	87.2
GA	28	25.2	14§	22.2	6	12.8
			p=0.65, NS, OR=0.85 (CI 0.41, 1.76)		p=0.08, NS, OR=0.43 (CI 0.17, 1.13)	
<i>Locus TNFA -238</i>						
GG	93	83.8	61	96.8	41	87.2
GA	18	16.2	2	3.2	6	12.8
			p=0.0095, ¶, OR=0.17 (CI 0.04, 0.76)		p=0.58, NS, OR=0.76 (CI 0.28, 2.04)	

*Number of subjects analysed.

†ES, Ewing's sarcoma.

‡In each polymorphism G is the normal allele and A is the polymorphic one.

§13 patients are GA and one is AA.

¶Statistically significant.

adult patients in the Tunisian population and the polymorphisms of *TNFA* are dependent on ethnicity.

In the present study, we report a decrease in the TNF238.2 rare allele among osteosarcoma paediatric patients and no difference from the control population in the distribution of the TNF308.2 variant in either of the tumour groups analysed, while other authors have described a decreased representation of the TNF308.2 allele in cancer populations (for example, in chronic lymphocytic leukaemia).¹⁹

Although the exact influence of the TNF238.2 variant on *TNFA* function and expression is not fully understood to date, the localisation of this polymorphism in the regulatory Y box²⁰ suggests that it may contribute to the optimal function and regulation of the *TNFA* promoter. However, it has been proven, in transfection assays, that there are no differences in *TNFA* production after stimulation of TNF238.2 heterozygous or normal homozygous cells; therefore TNF238.2 is not likely to be of functional relevance for transcriptional activation, and the actual meaning of the -238 promoter polymorphism remains a controversial matter.

There is evidence that the -308 TNF2 allele is over-expressed in diseases where *TNFA* levels are associated with poor prognosis. In our bone sarcoma series, we did not find a relationship between this or any other polymorphic variant of the *TNFA* promoter and tumour prognosis, disease free survival or development of metastasis or relapse (data not shown). One hypothesis is that these polymorphisms may serve as markers for additional polymorphisms or mutations in neighbouring genes that may be involved in the disease.

With regard to the increased number of *TNFA* polymorphic alleles in male osteosarcoma patients, several other authors have reported gender differences and increased *TNFA* levels in male patients affected by type II diabetes mellitus.²¹ A possible explanation for this finding is that the increased plasma levels of *TNFA* in males are the consequence of the presence of an increased number of the TNF308.2 allele, which has been associated with higher expression of the *TNFA* gene. In fact, in our series of osteosarcoma, there is a tendency for a higher number of TNF308.2 alleles in male patients, without reaching, however, statistical significance (p=0.079).

Computer DGGE programs Melt87 and SQTIX were kindly provided by Dr L. Lerman. We are also grateful to Dr H. Holden from the Laboratory of Molecular Medicine of the University of Sheffield for providing us with some polymorphic samples and to Dr Reyes López de Mesa for statistical assistance. This work was supported by a PIUNA grant from the University of Navarra.

ANA PATIÑO-GARCÍA*
ELENA SOTILLO-PINEIRO*
CONSUELO MODESTO†
LUIS SIERRASESÚMAGA*

*Laboratory of Paediatrics, University of Navarra and University Clinic, CIFA Building, Irúnlarrea SN, E31080 Pamplona, Spain

†Department of Internal Medicine, Hospital Val D'Hebrón, Barcelona, Spain

Correspondence to: Dr Patiño-García, apatigar@unav.es

- Rink L, Kirchner H. Recent progress in the tumor necrosis factor- α field. *Int Arch Allergy Immunol* 1996;111:199-209.
- Roesler K, Suchanek G, Breitschopf H, Kitz K, Matula C, Lassmann H, Koos WT. Detection of tumor necrosis factor- α protein and messenger RNA in human glial brain tumors. Comparison of immunohistochemistry with in situ hybridization using molecular probes. *J Neurosurg* 1995;83:291-7.
- Lejeune F, Lienard D, Eggermont A, Koops HS, Kroon B, Gerain J, Rosenkainer F, Schmitz P. Clinical experience with high-dose tumor necrosis factor α in regional therapy of advanced melanoma. *Circ Shock* 1994;43:191-7.
- Hermann SM, Ricard S, Nicaud V, Mallet C, Arveiler D, Evans A, Ruidavets JB, Luc G, Bara L, Parra HJ, Poirier O, Cambien F. Polymorphisms of the tumor necrosis factor- α gene, coronary heart disease and obesity. *Eur J Clin Invest* 1998;28:59-66.
- Hammann A, Mantzoros C, Vidal-Puig A, Flier JS. Genetic variability in the *TNFA* promoter is not associated with type II diabetes mellitus (NIDDM). *Biochem Biophys Res Commun* 1995;211:833-9.
- Ugliarolo AM, Turbay D, Pesavento PA, Delgado JC, McKenzie FE, Gribben JG, Hartl D, Yunis EJ, Goldfeld AE. Identification of three new single nucleotide polymorphisms in the human tumor necrosis factor- α gene promoter. *Tissue Antigens* 1998;52:359-67.
- Wilson AG, Symons JA, McDowell TL, McDevitt HO, Duff GW. Effects of a polymorphism in the human tumor necrosis factor α promoter on transcriptional activation. *Proc Natl Acad Sci USA* 1997;94:3195-9.
- Chouchane L, Ben Ahmed S, Baccouche S, Remadi S. Polymorphism in the tumor necrosis factor- α promoter region and in the heat shock protein 70 genes associated with malignant tumors. *Cancer* 1997;80:1489-96.
- Pociot F, D'Alfonso S, Compasso S, Scorza R, Richiardi PM. Functional analysis of a new polymorphism in the human TNF α gene promoter. *Scand J Immunol* 1995;42:501-4.
- Höhler T, Kruger A, Gerken G, Schneider PM, Meyer zum Büschenfelde KH, Rittner C. Tumor necrosis factor α promoter polymorphism at position -238 is associated with chronic active hepatitis C infection. *J Med Virol* 1998;54:173-7.
- McGuire W, Knight JC, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D. Severe malarial anemia and cerebral malaria are associated with different tumor necrosis factor promoter alleles. *J Infect Dis* 1999;179:287-90.
- Zheng MH, Wood DJ, Papadimitriou JM. What's new in the role of cytokines on osteoblast proliferation and differentiation? *Path Res Pract* 1992;188:1104-21.
- Jia SF, Kleiner ES. Antitumor activity of TNF α , IL-1 and IFN γ against three human osteosarcoma cell lines. *Lymphokine Cytokine Res* 1991;10:281-4.
- Van Valen F, Winkelmann W, Burdach S, Göbel U, Jürgens H. Interferon γ and tumor necrosis factor α induce a synergistic antiproliferative response in Ewing's sarcoma cells in vitro. *J Cancer Res Clin Oncol* 1993;119:615-21.
- Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
- Patiño-García A, Sotillo-Piñeiro E, Modesto C, Sierrasesúмага L. Screening of the tumor necrosis factor- α (TNF α) gene promoter polymorphisms by PCR-DGGE analysis. *Mutat Res Genomics* 1999;406:121-5.
- Wilson AG, di Giovane FS, Blakemore AF, Duff GW. Single base polymorphism in the human tumor necrosis factor α (TNF α) gene detectable by NcoI restriction of PCR product. *Hum Mol Genet* 1992;1:353.
- Verjans GM, Brinkmann BNM, Van Doornik CEM, Kijlstra A, Verweij CL. Polymorphism of tumor necrosis factor- α (TNF α) at position -308 in relation with ankylosing spondylitis. *Clin Exp Immunol* 1994;97:45-7.
- Demeter J, Porzolt F, Rämisch S, Schmidt D, Schmid M, Messer G. Polymorphism of the tumor necrosis factor- α and lymphotoxin- α genes in chronic lymphocytic leukaemia. *Br J Haematol* 1997;97:107-12.
- D'Alfonso S, Richiardi PM. A polymorphic variation in a putative regulation box of the *TNFA* promoter region. *Immunogenetics* 1994;39:150-4.
- Pfeiffer A, Janott J, Möhlig M, Ristow M, Rochlitz H, Busch K, Schatz H, Schifferdecker E. Circulating tumor necrosis factor- α is elevated in male but not in female patients with type II diabetes mellitus. *Horm Metab Res* 1997;29:111-14.

Low prevalence of germline *BRCA1* mutations in early onset breast cancer without a family history

EDITOR— Germline mutations in the *BRCA1* and *BRCA2* genes cause predisposition to breast and ovarian cancer.¹ Epidemiological evidence and linkage studies suggested that the likelihood that a woman with breast cancer has a genetic susceptibility to the condition is greater the younger she was at diagnosis and with increasing extent of family history of the disease. Studies of the prevalence of germline mutations in *BRCA1* and *BRCA2* in women with breast cancer has enabled the frequency of mutations to be determined in women with different ages at diagnosis and extent of family history of breast cancer.²⁻³ The CASH study into the attributable risk of breast and ovarian cancer estimated that 33% of all breast cancers diagnosed by the age of 29 years, and 22% diagnosed by the age of 30-39 years, are the result of an inherited mutation.⁴ However, the proportion of breast cancer cases diagnosed by 40 years resulting from a *BRCA1* mutation was predicted to be 5.3%.⁵ Previous population based studies of the prevalence of *BRCA1* mutations in early onset breast cancer have been in cases unselected for family history, and the majority of mutation carriers detected did have some degree of family history of either breast or ovarian cancer.⁶⁻¹⁰ The aim of this study was to establish the prevalence of *BRCA1* mutations in a large series of British patients with a young age of onset and no known family history of the disease, since such patients are referred relatively frequently for genetic counselling. The presence of *BRCA1* mutations in a significant proportion of these patients would have important implications for the planning of a mutation screening strategy in diagnostic services.

Patients were ascertained from the Imperial Cancer Research Fund Clinical Breast Oncology Department at Guy's Hospital, and the family cancer clinics at the Genetics Departments of Guy's Hospital and the Royal Free Hospital, both in London, and from St Mary's Hospital, Manchester. The Manchester cases were initially ascertained as part of a population based series for other studies. Patients without a known family history of breast or ovarian cancer at referral were recruited into this study, and these patients were interviewed at home by a genetic nurse counsellor. A family history was taken by the clinical geneticist or genetic nurse counsellor, and the diagnosis confirmed in all probands with either the referring breast unit or the patient's GP or oncologist. Patients with a known family history of breast or ovarian cancer at referral were excluded from the study. The range of age of diagnosis was 22-35 years and the median age of diagnosis was 31 years. Blood samples were taken after the purpose of the study had been explained and informed consent obtained. Full genetic counselling was provided, following agreed protocols, and results were made available to those women wishing to be informed.

The 22 *BRCA1* coding exons were amplified using 24 pairs of primers (exon 11 was amplified in three overlapping fragments). The fluorescent chemical cleavage of mismatch (FCCM) protocol was adapted from the method of Rowley *et al.*¹¹ PCR products were labelled by incorporation of dUTP analogues which were labelled with either R110 (blue), R6G (green), or Tamra (yellow)

fluorescent dyes (PE Applied Biosystems Inc), and heteroduplex molecules then subjected to hydroxylamine modification and piperidine cleavage. Using the three different dyes to label the 24 PCR products that cover the complete *BRCA1* coding sequence, three patients could be analysed for one fragment in one lane of the gel; thus three patients were completely screened in 24 lanes and six patients were screened on a 50 well gel. The FCCM technique has been reported to detect over 95% of mutations in a blind study of haemophilia A patients.¹¹ We evaluated the sensitivity of our *BRCA1* protocol by examination of the eight polymorphisms that have been reported in the *BRCA1* gene with a frequency of at least 5% (1186A/G, 2201C/T, 2430T/C, 2731C/T, 3232A/G, 3667A/G, 4427C/T, 4956A/G).¹² These polymorphisms were all detected reproducibly at the expected frequency in a panel of over 400 patients who were tested, as part of our continuing studies. In addition, FCCM detected the mutations in three known *BRCA1* positive samples (188del 11bp, 5242 C/A, and 5382insC) that were previously found in our laboratory by SSCP analysis.¹³ The fluorescent chemical cleavage assay which we have developed therefore allows a rapid and sensitive mutation screen of *BRCA1*.

Four mutations that were likely to be pathogenic were detected in 110 patients (3.6%) and are listed in table 1. These included three sequence variants that would be predicted to result in premature termination of translation. The 185delAG frameshift mutation, which is prevalent in the Ashkenazi Jewish population, was identified in a British patient (91032) with Jewish ancestry. The 4693-4694delAA mutation was detected in a British patient (78750) who was diagnosed with breast cancer at the age of 26 years. The truncating mutation 3875-3878delGTCT was seen in a patient of Afro-Caribbean origin (94641), who was diagnosed with breast cancer aged 33 years and with a second primary cancer at 38 years. Testing for this mutation in the parents of the patient indicated that it was inherited from her father. A novel in frame deletion, 1965-1967delTCT, was detected in a patient of West African origin (103727) who was diagnosed with breast cancer at the age of 27 years. This mutation would be predicted to result in the deletion of a single amino acid, serine 616, but would not lead to premature termination of translation. This sequence change was absent in over 350 control chromosomes in our study and fulfils all the other criteria for pathogenic status.¹³ The pedigrees of these four women are shown in fig 1. Although all four patients originally reported no family history of breast cancer, further investigation showed that the maternal great grandmother of patient 103727 was diagnosed with the disease at about 60 years of age. The lack of a history of breast or ovarian cancer in these families is likely to result from a combination of factors including paternal transmission of the mutation, chance, and reduced penetrance.

Eight other rare DNA sequence variants were identified (table 2). Three of these would not be predicted to alter the expression of *BRCA1* or the sequence of its encoded protein (Q1604Q, IVS22+8T/C, UGA+36T/C) and R1347G

Table 1 *BRCA1* pathogenic mutations

Patient	Exon	Nucleotide change	Amino acid change
91032	2	185-186delAG	39 stop
103727	11	1965-1967delTCT	S616del
94641	11	3875-3878delGTCT	1264 stop
78750	15	4693-4694delAA	1529 stop