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A GENOTYPE-PHENOTYPE STUDY

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We performed a prospective genotype-phenotype study using molecular screening and clinical assessment to compare the severity of disease and the risk of sarcoma in 172 individuals (78 families) with hereditary multiple exostoses. We calculated the severity of disease including stature, number of exostoses, number of surgical procedures that were necessary, deformity and functional parameters and used molecular techniques to identify the genetic mutations in affected individuals. Each arm of the genotype-phenotype study was blind to the outcome of the other. Mutations *EXT1* and *EXT2* were almost equally common, and were identified in 83% of individuals. Non-parametric statistical tests were used.

There was a wide variation in the severity of disease. Children under ten years of age had fewer exostoses, consistent with the known age-related penetrance of this condition. The severity of the disease did not differ significantly with gender and was very variable within any given family. The sites of mutation affected the severity of disease with patients with *EXT1* mutations having a significantly worse condition than those with *EXT2* mutations in three of five parameters of severity (stature, deformity and functional parameters). A single sarcoma developed in an *EXT2* mutation carrier, compared with seven in *EXT1* mutation carriers. There was no evidence that sarcomas arose more commonly in families in whom the disease was more severe.

The sarcoma risk in *EXT1* carriers is similar to the risk of breast cancer in an older population subjected to breast-screening, suggesting that a role for regular screening in patients with hereditary multiple exostoses is justifiable.

Hereditary multiple exostoses (HME), also known as multiple hereditary exostoses, multiple osteochondromatosis or diaphyseal aclasis, is an autosomal dominant inherited trait with an incidence of at least one in 50 000, which makes it one of the most common inherited musculoskeletal conditions.[1] Multiple cartilage capped exostoses develop during childhood and adolescence in the metaphyseal region of long bones, resulting in short stature and deformity, often requiring corrective surgery.[1,2] Most lesions stagnate and ossify when skeletal growth is complete, but occasionally one might grow more aggressively or reactivate as a chondrosarcoma.[3-5] The severity of disease in HME varies considerably. Some individuals achieve normal height with few exostoses, whereas others are badly affected. Radiological studies confirm this variation, yet the factors that determine severity of the disease are not known.[1,2]

It is possible that some, or all, of the variation is due to genetic differences. In conditions such as achondroplasia, a single gene is affected in the same way in all patients. In patients with HME, two different genes, each on different chromosomes, are associated with the condition. It has also been shown that a variety of mutations occur in these genes in HME. It has been suggested that genetic variation could account for the difference in severity of the disease.[6-11] Knudson[12] demonstrated a mathematical relationship between observed numbers of tumours and cellular mutation rates in familial neoplastic traits. The risk of malignant change might also be related to osteochondroma load and severity of the disease. Therefore, we designed a prospective study to assess the risk of sarcoma formation and the variation in severity with different genetic mutations, to determine whether a particular mutation had a morbid effect.

Patients and Methods

Our prospective study comprised 172 individuals (78 families) with HME. The researchers

collecting the genotype data were 'blind' to the results of the phenotype data, and vice versa. Index patients with solitary osteochondromas were excluded.

The phenotype can be characterised in several ways. Most relevant to disease activity is the number of exostoses, especially as genetic epidemiology predicts this to be a major determinant of the risk of sarcoma.[12,13] Assessing the number of osteochondromas by clinical examination alone has previously been shown to correlate with radiological data,[2] suggesting that in population studies, clinical examination provides a reliable estimate of the severity of disease and avoids the theoretical risk of malignant change associated with ionising radiation.[14,15]

Population-based studies have defined some deformities which are inherent in HME. These include short ulnae, dislocated proximal radio-ulnar joint, forearm bowing, genu vara/valga and short stature generally.[1] Handicap, however, is reflected in functional parameters, as well as anatomical ones. It is recognised that some osteochondromas have greater disabling effects than others (such as those close to the distal ulnar epiphysis) and an assessment of the disease phenotype has to take this into account.[2] Disability (functional effects) include reduced range of movement of elbow, forearm and knee. The severity of these deforming and functional features of HME are graded in Table I. Standing height was measured and compared with age and gender-matched data from Buckler-Tanner.[16] The number of exostoses, the number of previous surgical operations and the magnitude of the deforming and functional effect of osteochondroma were compared in separate genotype-phenotype analyses.

Patients were selected by referral from orthopaedic surgeons, geneticists and occasionally they presented themselves, between 1996 and 2000. All had the clinical manifestations of HME.

All patients were informed of the programme and agreed to take part. Ethical approval was obtained for sample collection and analysis. A clinical history was obtained and a family pedigree. The clinical data was collected by clinical examination carried out by a single examiner (MF) who was blind to all genotypic data.

Genomic DNA was extracted either from blood, using the Nucleon DNA extraction Kit (Scotlab, Lanarkshire, UK), or from buccal swabs by a previously described protocol.[10] The lab investigator was blind to phenotypic data. The initial mutational analysis of *EXT1* and *EXT2* was carried out by conformation-sensitive gel electrophoresis and fluorescent single-strand conformation polymorphism analysis, but the greater part of the study was carried out using denaturing high-performance liquid chromatography (DHPLC).[10,11] *EXT1* and *EXT2* exons and their flanking intronic regions were amplified by polymerase chain reaction (PCR) from genomic DNA isolated from proband samples by the use of specific primer pairs. For DHPLC analysis the initial primer pairs used were as previously

Table I. Severity scoring criteria for deformity and functional scores in HME disease

Criterion	Severity	Score
Deformity		
Ulnar length as a proportion of height (%)	>14	0
	13 to13.9	1
	12 to12.9	2
	<12	3
Forearm deformity	None	0
	Ulnar negative variance without bowing	1
	Forearm bowing (no radial head dislocation)	2
	Radial head dislocation	3
Knee deformity (°)	None	0
	Genu vaga 5 to10	1
	Genu vaga 10 to15	2
	Genu vaga >15	3
		Range 0 to 18 (bilateral assessment)
Functional		
Forearm range of rotation (°)	>180	0
	160 to 179	1
	140 to 159	2
	<140	3
Elbow range of flexion (°)	>140	0
	130 to 139	1
	120 to 129	2
	<120	3
Knee range of flexion (°)	>130	0
	120 to 129	1
	110 to 119	2
	<110	3
		Range 0 to 18 (bilateral assessment)

ment Analysis System (Transgenomic, Cheshire, UK).[11] The full sequence of the PCR product for all fragments were entered into the Transgenomic WAVEMaker 3.4 program (Transgenomic). This enabled the temperature and gradient conditions for each fragment to be selected. The crude PCR products, which had been denatured, followed by gradual re-annealing, were injected into a DNASep column (Transgenomic). The column mobile phase consisted of a linear acetonitrile gradient in a 0.1 M triethylamine acetate buffer (TEAA). The calculated gradient, at a flow rate of 0.9 ml/min, was run for all the amplicons at the relevant column temperature for each fragment.

Where a variant chromatograph was obtained, the fragment was amplified by PCR using the same primers and the DNA sequence determined. The PCR product was purified through a spin column, using a QIAquick PCR purification kit (Qiagen, Crowley, UK), then sequenced in both directions using BigDye Terminator Cycle Sequencing Ready Reaction kit (PE-Applied Biosystems, Warrington, UK) and run on an ABI 377 DNA Sequencer (PE-Applied Biosystems). DNA from both affected and unaffected family members was sequenced.
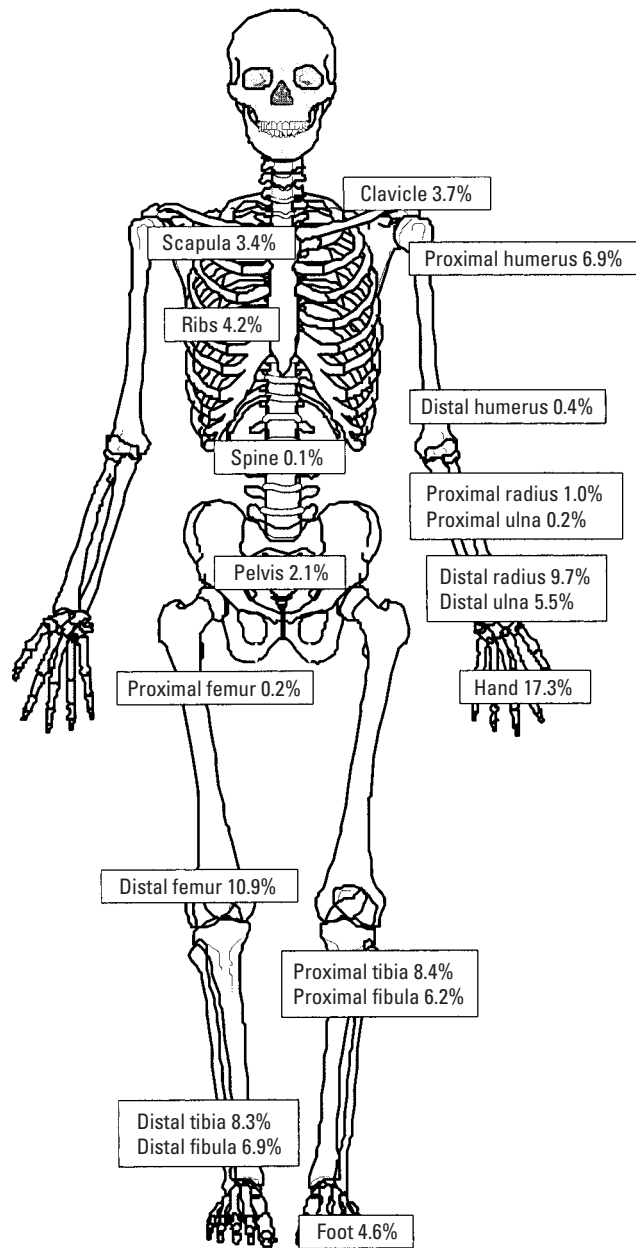
Fig. 1

Percentage of palpable exostoses found in the 172 individuals with HME by anatomical site.

Statistical analysis. Individuals from the same family cannot be viewed as truly independent of each other. Therefore, except for analyses of age and gender, all analyses were undertaken on the mean value of the numbers of exostoses, deformity and functional grading, in each family. No matter how many family members were assessed, only one datum point was generated for each family. Statistical analysis of all data was undertaken using non-parametric tests (chi-squared, Wilcoxon rank-sum test, and Kendall's test of rank correlation).

Results

Between 1996 and 2000, we assessed the genotype and phenotype of 172 individuals from 78 families with HME. Genotyping revealed 71 individuals (34 families) with *EXT1* mutation, 72 (27 families) with *EXT2* and 29 (17 families) with no identifiable mutation. The majority (50 of 61 families with known mutations) were loss-of-function mutations.

The number of palpable exostoses varied considerably from site to site. Their anatomical frequency in our series is recorded in Figure 1. Age-distribution is shown in Figure 2. The number of exostoses in individuals ranged from zero to 172, but did not demonstrate a significant correlation with age (rank correlation coefficient = 0.027, p = 0.296). A trend towards fewer exostoses, however, was seen in children up to the age of ten years (Fig. 2) who were almost equally represented in both *EXT1* (n = 18) and *EXT2* (n = 16) mutation groups.

There were 82 men and boys and 90 women and girls. There was a trend towards more severe disease in the men and boys than in the women and girls, but this failed to reach statistical significance in the several parameters of severity which were assessed (median number of exostoses 27 and 22, respectively; z = 1.840, p = 0.066). Boys and girls were almost equally represented in the group aged less than ten years. Gender was not significantly different between families with *EXT1* and *EXT2* mutations.

Definite loss-of-function mutations were found in 50 families and missense mutations in 11. In 17 families no mutation could be identified. When the numbers of exostoses were compared in families with loss-of-function and missense mutations, no significant difference was noted (median number of exostoses 29 and 24, respectively; z = 0.993, p = 0.321). Of 14 new unpublished mutations in our data-set, 13 were loss-of-function and one a missense mutation.

Families with an *EXT1* mutation had significantly worse disease than those with an *EXT2* mutation across several parameters of severity (Fig. 3); 19 of 34 families (56%) with *EXT1* consisted of individuals whose mean height was below the 25th centile, compared with six of 27 individuals (22%) in families with *EXT2* (X^2 = 7.050, p = 0.008). The median family height in families with the *EXT1* mutation was on the 18th centile, and that for families with the *EXT2* mutation was on the 50th centile. Individual deformity scores ranged from zero to 17 from a total of 18. Significantly greater deformity scores were recorded in families with the *EXT1* mutation than those with *EXT2* (median score 5.0 and 1.8, respectively; z = 3.532, p = 0.0004). Individual functional scores ranged from zero to 16 from a total of 18. Significantly worse functional scores were recorded in families with the *EXT1* mutation than those with *EXT2* (median score 6.0 and 3.1, respectively; z = 2.512, p = 0.012).

The number of exostoses per individual varied between zero and 172. The number of exostoses were similar in
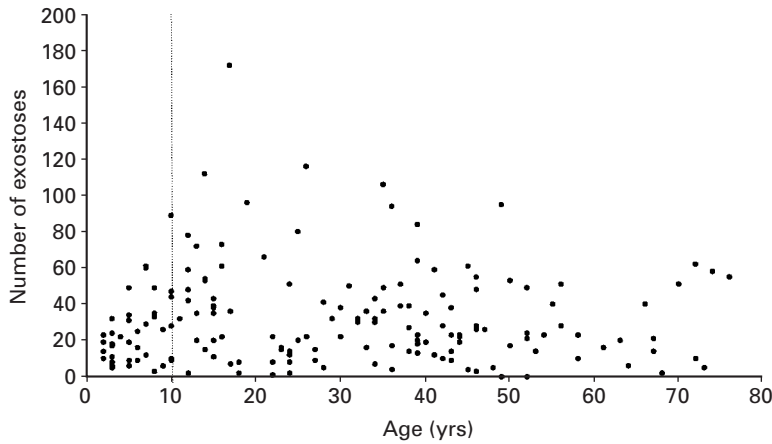
Fig. 2

Number of exostoses by age demonstrating age-dependent phenotypic penetrance up to the age of ten years.
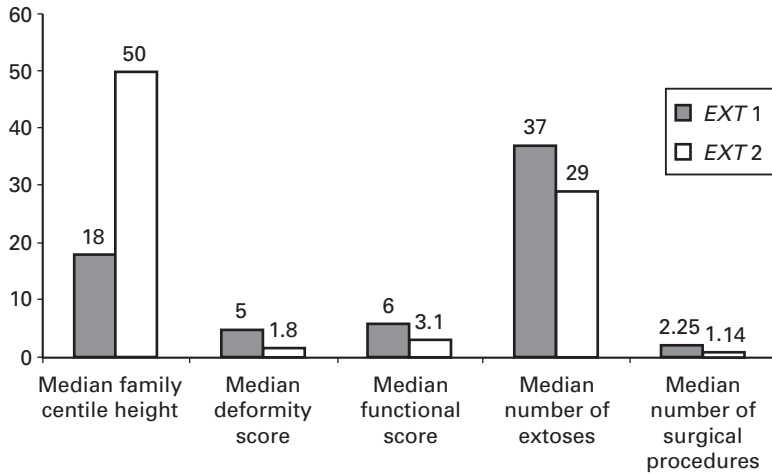

Fig. 3

Parameters of disease severity of HME in families with *EXT1* and *EXT2* mutations.

Table II. Profile of chondrosarcomas in individuals affected with HME

	EXT1 mutation	EXT2 mutation	No mutation identified	Site
Sarcoma in examined individual	6	0	1	2 x scapula, 2 x spine, pelvis, rib, proximal humerus
Sarcoma reported in relative	1	1	1	2 x pelvis, femur
Total	7	1	2	

families with *EXT1* and *EXT2* mutations (median 27 and 29, mean 37 and 29, respectively; $z = 1.430$, $p = 0.153$).

A total of ten chondrosarcomas occurred in nine families; seven in subjects within the study and three in relatives who had already died (Table II). All sarcoma patients inherited loss-of-function mutations in their defective *EXT* gene; seven sarcomas occured in *EXT1* mutation carriers, and only one in an *EXT2* mutation carrier. No statistical difference was identified when the number of exostoses was compared in families with or without a family history of sarcoma (median of exostoses 37 and 28, respectively; $z = 0.962$, $p = 0.336$).

Discussion

Both epidemiological and clinical features of this condition allow its classification as a familial (mostly benign) neoplastic trait.[2,13] This assertion is strengthened by molecular genetic evidence which strongly suggests a clonal aetiology
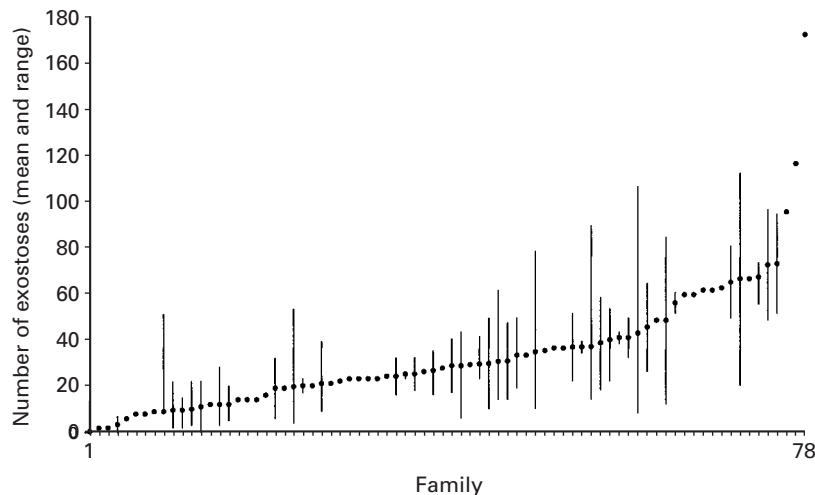
Fig. 4

Number of exostoses (mean and range) for each family with HME.

for the osteochondroma and that a 'two-hit' model for HME pathogenesis is appropriate. Mutations of the germ line in the *EXT* tumour suppressor gene family have been identified in HME.[6-10,12,17-22] The risk of sarcoma in HME is estimated, in population-based series, to be smaller than initially thought. Recent estimates would suggest a lifetime risk of about 2% to 4%.[3,23-25] In published series, the *EXT1* mutation has been identified in about 45% of HME patients and about 40% in the *EXT2*. The remaining 15% have no identified mutation of the germ line.[6-11] In our group, the ratio of families with *EXT1* and *EXT2* mutations is similar to those in other published series, both self-selected and hospital-based.[8,10,11] The anatomical distribution is also similar to published radiological skeletal surveys, although the numbers identified close to deep-seated joints (proximal femur, pelvis) are lower.[1,5,25,26]

Our results confirm an age-related phenotypic penetrance. This is widely acknowledged in HME and consistent with published data, which report an age-dependent disease penetrance and a median age of first diagnosis of HME[5] of three years.[1] A trend towards worse disease was observed in men and boys. These results are consistent with other studies and suggest only a limited role for the disease-modifying effects of gender.[1,5]

Although loss-of-function mutations result in no useful gene-product, missense mutations (substitution of one amino acid for another) might allow partial gene-function if the mutation did not occur at a critical site. Our data reveal that the majority of *EXT* mutations are associated with loss-of-function, which would be expected in a tumour-suppressor gene, requiring knock-out for disease-expression. Furthermore, most missense mutations (10 of 11) have been previously described,[27] suggesting that they occur in crucial sites causing an effective 'knock-out' of the gene. Consequently, similar disease severities might be expected.

The findings of an earlier smaller genotype-phenotype study in HME concur with our results.[28] Our group of patients is considerably larger, enabling the variables of age and gender to be examined. *EXT1* mutation is found more frequently than *EXT2* mutation in solitary exostoses, but it is not yet known why this gene has a more dominant role in severity of disease and sarcoma-risk in HME.[22] It is likely that a large non-*EXT* gene component to severity of disease exists since family members frequently exhibit a wide range of HME scores (Fig. 4).

In regard to the association between the *EXT1* mutation and chondrosarcoma, three questions arise; is it worth screening for pre-malignant disease, and if so, how and in whom? Given a lifetime risk of approximately 3%, most of which occurs in the third to fifth decades of life, it would seem reasonable to assess the annual risk of sarcoma in this age group to be 0.1%.[3,22-24] Possibly this risk might be doubled in *EXT1* mutation carriers. By comparison, the incidence of breast cancer is 0.2% per annum between the ages of 50 and 60 years;[29] an age group in which mammographic screening is recommended in many countries. By this comparison, a screening programme in HME (in particular *EXT1* mutation carriers) might seem to be appropriate.

Current practice is in favour of clinical examination once every year or two. There are no data on the number of malignancies that develop between screening visits, nor on the efficacy of such a programme. In our series only 13.5% of palpable exostoses were found in the axial skeleton (Fig. 1), while in a large recent report of chondrosarcomas in HME, 80% of tumours occurred in the axial skeleton.[30] Worries, therefore, exist as to whether deep-seated axial exostoses or chondrosarcomas can be identified by clinical examination alone. Radiological surveys deliver a significant radiation dose, and have theoretical risks in a condition now identified as a familial neoplastic trait. Magnetic

resonance imaging of the axial skeleton is a promising line of investigation in screening and positron-emission tomography has also been used to study exostosis activity. Both imaging modalities may have a role in screening, however, no published data on their efficacy exist.

Given that families with a history of sarcoma do not have a significantly greater median HME score than those with no such history, it seems that severity of disease alone cannot be utilised as a marker of the risk of malignant change. *EXT* gene testing may have a role, but at least one sarcoma has been reported in an *EXT2* mutation carrier,[21] in addition to the patient reported here. Thus, all known patients with HME may benefit from inclusion in a screening programme.

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