Somatic mutations of KIT in familial testicular germ cell tumours

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Testicular germ cell tumours (TGCTs) are the most common malignancy in males between the age 15 and 45 years (Ferlay et al., 2001). There are several risk factors for TGCT including previously diagnosed TGCT, undescended testis (UDT) and a family history of the disease. TGCT has been one of the highest familial relative risks of any cancer syndrome with reported increased risks of 8–10-fold to brothers and 4–6-fold to fathers (Forman et al., 1992; Heimdal et al., 1996). We previously described linkage of familial testicular cancer to a locus (TGCT1) at Xq27 (Rapley et al., 2000). This locus was particularly strongly associated with families characterised by at least one case of bilateral testicular cancer. The results indicated, however, that only a minority of families are attributable to this locus and that additional TGCT susceptibility genes are likely to exist.

The KIT gene encodes a type III transmembrane tyrosine kinase receptor. KIT is expressed in several cell types where it regulates primordial germ cell migration, proliferation and apoptosis during foetal gonad development (Mauduit et al., 1999). KIT has been shown to be expressed in some TGCT (Strohmeyer et al., 1995; Bokemeyer et al., 1996) and somatic mutations in KIT have recently been identified in testicular (Tian et al., 1999) and mediastinal germ cell tumours (Przygodzki et al., 2002). Mutations have been reported in a high proportion of patients with bilateral disease, and in a much smaller proportion of unilateral cases (Looijenga et al., 2003). When both tumours from bilateral cases could be examined,
the same mutation was present in both tumours. Together, these results suggest that somatic KIT mutations occur early in embryogenesis, before the primordial germ cells have divided and migrated to the gonads. As a consequence, primordial germ cells with KIT mutations are distributed to both testes and hence KIT mutations are associated with bilateral disease (Looijenga et al, 2003).

Previous studies have indicated that KIT mutations found in germ cell tumours are somatic. To investigate further the role of KIT in predisposition to TGCT, and the role of somatic mutations in familial tumours, we have examined a series of constitutional and tumour DNAs from patients with TGCTs and a family history of the disease.

MATERIALS AND METHODS

The International Testicular Cancer Linkage Consortium (ITCLC) has obtained samples from 326 families with two or more cases of TGCT (Table 1). For this study, we analysed DNA extracted from blood lymphocytes (constitutional DNA) from one affected individual from each of 240 families, for whom the DNA was most readily available. The pedigree structure for these families is shown in Table 1. We also examined tumour materials from 123 cases from 93 families; for 15 of these families, the tumour material only was available and these were therefore not included in the 240 constitutional DNA set (Table 1). Seven tumours were from patients with bilateral disease, but the tumour material was only available from one of each pair of tumours arising in these patients.

Patients donated samples and medical information with full informed consent and with local or national ethical review board approval. Information on clinical status including type of TGCT, age of diagnosis, presence of UDT and laterality of disease was confirmed by reviewing histological reports and clinical notes.

DNA was prepared from whole blood and from formalin-fixed, paraffin-embedded tumour sections using standard techniques. The tumour material was microdissected to minimise contamination by surrounding normal tissue. Primer sequences for KIT were designed from the KIT mRNA and genomic sequence (Ensembl

| Table 1 | Pedigree structure of cases with family history of TGCT used in KIT mutation search |
|---------|----------------------------------------------------------------------------------|
| **Family type** | **Pedigrees (by type) identified by ITCLC** | **Number of pedigrees analysed (by type) using constitutional DNA** | **Number of pedigrees analysed (by type) using DNA from tumour material** |
| Sib trios | 8 | 5 | 2 |
| Large >= 3 affected cases | 31 | 22 | 3 |
| Sib pairs | 154 | 106 | 43 (11)* |
| Father/son pairs | 52 | 46 | 16 (2)* |
| Cousin pairs | 40 | 33 | 14 |
| Uncle/nephew pairs | 32 | 23 | 13 (2)* |
| Grandfather/grandson pairs | 4 | 5 | 1 |
| Monozygous twins | 4 | 0 | 0 |
| Great grandfather/great grandson pairs | 1 | 0 | 1 |
| **Total** | **326** | **240** | **93** |

*Number in brackets represents pedigrees (by subtype) for which only tumour material was available for analysis, these families are not included in the KIT analysis of constitutional DNA samples.

| Table 2 | KIT gene primer pairs |
|---------|-----------------------|
| **Exon** | **Forward** | **Reverse** | **PCR product size** |
| Exon2a | AATAGCCAGGGCACTTGTGC | GTTTGGTCCAGTCTATTTGC | 358 |
| Exon2b | CTTCGAGCTCCTCTCTCAAC | CACTTCTAGACCCACGCAAG | 395 |
| Exon3a | GTGGCGTAGCAGCGAAAC | GTGAGCGGTGTTCTACACTT | 397 |
| Exon3b | GCTTCTATAGCTGCCACAGC | AGGTTGATCAACAGAAGAGA | 372 |
| Exon4 | GATGGTCTAGCCACCTGTGT | TCTCCCAGAACATCCACTC | 400 |
| Exon5 | TGGAGAATTTAATGCTCTATTTT | TCATTCTATTGATAGAAACATT | 389 |
| Exon6 | GGAAATCGAACTACTTGGTTT | TCCTGGAATTAGGGTTTAC | 384 |
| Exon7 | CTTCCAAGCAAGCATAGTTTCC | AACCACCAAAACGCAGTTC | 364 |
| Exon8 | TTCTGCCCCCTTGAATGTGT | AAAGCCACATGGCTAGAAA | 386 |
| Exon9 | ATGCCACATCCCAATGGTT | TGCAGTGGTCAATGGGAAA | 364 |
| Exon10 | AACCAGGAGTGAACTCTGAGAC | CTCCTCAACAAACCTTCAGT | 384 |
| Exon10 small | ATCCACCTCTGCCAAATTT | CTGTTGGGAAAGAAG | 246 |
| Exon11 | TAGGTGCGATGATTGCCATT | GGGCAGATTTACCAGAAAAC | 397 |
| Exon11 small | AGAAGTGGCTCTAAAGCTGAGCACA | AAAACAGGAGAACACTTGGA | 279 |
| Exon12 | ATTGGGGCCATCTTTATG | GTCTGAGCACTGGGTTTCC | 392 |
| Exon13 | TGCTAACAGCTAAGGGCTT | GCAAGAGGAAACAGAAGCTTG | 335 |
| Exon14a | CTCACACATAGGGCTCTT | CCCATGAACTCTCCCAGTAC | 381 |
| Exon14b | TTCTCACTTTTTCTTCTACCTTTT | TCAGCACAATTCTAGGTTTACAT | 390 |
| Exon15 | TGTGACGAAAGGGATGAGGA | CCCTACTCTGCGCTGACAT | 335 |
| Exon16 | GATCTGCTGCTGCAAACTTCCA | AAAAAACCCATCTTTAGAAGATC | 385 |
| Exon17 | CACATTCAAGCCGGATTTTGG | TCCCTAGACAGGATATCATTTGAG | 399 |
| Exon17 small | TAAATTGGCTCTTCTTCTCTCCCA | TTTCTTGAAAATTCTTTTGCTGG | 233 |
| Exon18 | CTTACAGTTCTGAGCAACG | GGCCTCTTCTCGACACAC | 333 |
| Exon19 | AAGTGTTGGCCACCTGAAA | CCCCTCAACATGCGTGTTTCT | 390 |
| Exon20 | TCCATATGAGTTCTGACAGC | GCCCAATTTGCAACACTTTAGA | 350 |
| Exon21 | TTTCCATCCTGTTGTTGCTT | GAACAAAAATCTAGGGCCACT | 390 |
gene ID = ENSG00000157404), using the Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Primer sequences for the 21 KIT exons and PCR product sizes are shown in Table 2. Exons > 400 bp were amplified using overlapping primer pairs. A total of 23 primers pairs were used to examine the coding region of the KIT gene. Primers designed for exon 1 failed to amplify under a variety of PCR conditions and were redesigned but again failed to give a PCR product. Exon 1 was therefore not examined. Mutations of KIT are predominantly located in exons 10, 11 and 17 (Pignon et al, 1997; Tian et al, 1999; Rubin et al, 2001; Przygodzki et al, 2002; Looijenga et al, 2003); therefore, the tumour material was examined only at these exons. Primers generating a smaller sized PCR fragment were designed for exons 10, 11 and 17 to allow easy amplification from paraffin-embedded material and were specifically used to analyse the tumour material.

For constitutional DNA, all exons were examined by conformation sensitive gel electrophoresis (CSGE) (Ganguly et al, 1993). Briefly, both PCR primers were labelled with adenosine 5’-[γ-32P]triphosphate by T4 polynucleotide kinase. After amplification, PCR products were heated to 98°C and cooled down to 60°C over 30 min to allow heteroduplex formation. PCR samples were run on a CSGE gel (10% v/v acrylamide with 4 mg/ml 1 × GTB buffer (89 mM Tris, 29 mM taurine and 0.5 mM EDTA). Polymerase chain reaction (PCR) products from samples that showed migration shifts on CSGE were bidirectionally sequenced using the BigDye terminator kit and a 3100 automated sequencer (Applied Biosystems, Warrington, UK).

All tumour samples were examined for exons 10, 11 and 17 of the KIT gene by direct sequencing. The tumour material was amplified and sequenced using specifically designed primers that generated a smaller PCR fragment than those designed for CSGE analysis and allowed for ease of amplification from tumour material. Sequencing was performed using the BigDye terminator v3 sequencing kit and a 3100 automated sequencer (Applied Biosystems).

Differences in distribution between categorical variables were assessed with the appropriate contingency table test.

RESULTS AND DISCUSSION

In total, 240 constitutional DNA samples from TGCT cases with a positive family history were examined using CSGE. Two conservative nonsynonymous constitutional sequence variants were detected, M541L and V399I (Table 3). M541L was found in 43 out of 240 (17.9%) cases and it is a common polymorphism found in 32 out of 192 (16.6%) of normal controls. V399I was found in only a single case and was not found in 200 controls. V399I is not conserved in other species (mouse, zebrafish and xenopus) and the amino-acid substitution is conservative, suggesting that this variant is a rare polymorphism rather than a disease-causing change. Overall, the results provide no evidence that germline KIT mutations are associated with an increased risk of testicular cancer.

Somatic mutations of KIT were detected in five out of 123 TGCTs examined (Table 4 and Figure 1). Three mutations involved codon 816, a known hotspot for KIT mutations in testicular (Tian et al, 1999; Przygodzki et al, 2002; Looijenga et al, 2003) and other cancers (Rubin et al, 2001). Another mutation involved codon 820, an infrequently mutated residue but one that has been previously reported (Pignon et al, 1997). The fifth mutation was a 12 bp deletion encoding a 4 amino-acid in-frame deletion in the cytoplasmic juxamembrane domain of KIT. In-frame deletions of this region are common in gastrointestinal stromal tumours (GIST) (Rubin et al, 2001) but have not been documented in TGCT. All these mutations were shown to be somatic. Two out of seven (28.5%) familial bilateral cases carried a somatic KIT mutation compared with three out of 116 (2.6%) familial unilateral cases (P = 0.026, Fisher’s exact test). Unfortunately, samples of the other tumour/ITGCN from the two bilateral cases with KIT

### Table 3 Sequence variants in KIT detected in constitutional DNA from patients with familial TGCTs

| Exon | Nucleotide change RefSeq NM_000222 | Amino-acid change | Number of cases |
|------|-----------------------------------|-------------------|-----------------|
| 3    | G525A                             | A168A             | 1               |
| 6    | C999T                             | N326N             | 1               |
| 7    | G1216A                            | V399I             | 1               |
| 10   | A1642C                            | M541L             | 43              |
| 10   | A1659G                            | K546K             | 5               |
| 16   | C2370T                            | L783L             | 1               |
| 17   | C2451T                            | I798I             | 12              |
| 18   | G2607C                            | L862L             | 53              |
| 19   | G2643A                            | P874P             | 1               |

### Table 4 Sequence changes in KIT detected in TGCTs

| Sample name | Exon | Nucleotide change RefSeq NM_000222 | Amino-acid change | Case details giving tumour type, age at diagnosis, history of undescended tests and family history |
|-------------|------|-----------------------------------|-------------------|--------------------------------------------------------------------------------------------------|
| 2158-201    | 11   | Del 1675–1686                     | del MYEV AA 552–555 | R. sem* and ITGCN                                                                                    |
|             |      |                                   |                   | Age at diagnosis = 39 years                                                                       |
|             |      |                                   |                   | History of bilateral UDT                                                                             |
|             |      |                                   |                   | Family history = sib pair                                                                            |
|             |      |                                   |                   | L. sem                                                                                             |
|             |      |                                   |                   | Age at diagnosis = 52 years                                                                         |
|             |      |                                   |                   | Family history = MZ twins (twin brother has bilateral disease, no tumour available for this patient)|
| 274-201     | 17   | A2480G                            | D820G             | R. mixed and L. sem                                                                                  |
|             |      |                                   |                   | Age at diagnosis = 39 and 55 years                                                                  |
|             |      |                                   |                   | History of UDT                                                                                    |
|             |      |                                   |                   | Family history = sib trio                                                                             |
| 295-304     | 17   | G2467C                            | D816H             | R NS                                                                                              |
|             |      |                                   |                   | Age at diagnosis = 66 years                                                                         |
|             |      |                                   |                   | Family history = father/son pair                                                                     |
| 377-1664    | 17   | G2467T                            | D816Y             | L. sem                                                                                              |
|             |      |                                   |                   | Age at diagnosis = 39 years                                                                         |
|             |      |                                   |                   | Family history = sib pair                                                                            |

*Tumour examined in bilateral cases. Sem = Seminoma; NS = nonseminoma; ITGCN = intratubular germ cell neoplasia; R = right side; L = left side; UDT = undescended tests.
mutations were not available to evaluate the presence of the mutations. While the frequency of KIT mutations in unilateral TGCT is similar to that detected previously, the proportion of cases with bilateral disease is much lower despite the fact that we examined a larger proportion of the KIT gene than in the study by Looijenga et al (2003). The reason for this is unclear. However, it may indicate that bilateral disease in the context of familial testicular cancer has a different pathogenesis from sporadic bilateral cases and that most of the familial bilateral cases are explained by the elevated risk conferred by the underlying susceptibility genes. Nevertheless, the overall pattern of an elevated frequency of KIT mutations in bilateral compared to unilateral cases supports the observation of Looijenga et al (2003) and suggests that somatic KIT mutations may take place early in development.

In conclusion, our results indicate that constitutional mutations of KIT are not associated with a substantially increased risk of TGCT. Somatic mutations of KIT are found in familial TGCT tumours with a higher proportion in cases with bilateral disease. Overall, the proportion of KIT mutations in TGCT is low and other somatic and susceptibility genes must play important roles.

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