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Analysis of the *DND1* Gene in Men with Sporadic and Familial Testicular Germ Cell Tumors

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Abstract

A base substitution in the mouse *Dnd1* gene resulting in a truncated Dnd protein has been shown to be responsible for germ cell loss and the development of testicular germ cell tumors (TGCT) in the 129 strain of mice. We investigated the human orthologue of this gene in 263 patients (165 with a family history of TGCT and 98 without) and found a rare heterozygous variant, p. Glu86Ala, in a single case. This variant was not present in control chromosomes (0/4,132). Analysis of the variant in an additional 842 index TGCT cases (269 with a family history of TGCT and 573 without) did not reveal any additional instances. The variant, p. Glu86Ala, is within a known functional domain of *DND1* and is highly conserved through evolution. Although the variant may be a rare polymorphism, a change at such a highly conserved residue is characteristic of a disease-causing variant. Whether it is disease-causing or not, mutations in *DND1* make, at most, a very small contribution to TGCT susceptibility in adults and adolescents.

INTRODUCTION

Testicular Germ Cell Tumor (TGCT) is the most common cancer in males aged 15–45 years. Among the known risk factors, a family history of disease is one of the strongest and most consistent (Forman et al., 1992; Heimdal et al., 1996). The relative risk to a brother of a TGCT patient is between 8 and 10 (Forman et al., 1992; Heimdal et al., 1996; Hemminki and Li, 2004). This familial relative risk is substantially higher than for most other cancer types, which are typically between two and four (Dong and Hemminki, 2001). Several lines of evidence indicate that genetic susceptibility genes are important in this disease (Holzik et al., 2004).

The largest genome-wide linkage study for TGCT suggested that susceptibility arises via multiple genes with modest effects on risk and that no major gene can explain a large fraction of the familial risk (Crockford et al., 2006). Recently, a deletion on the Y chromosome characterized as gr/gr has been associated with a twofold risk of developing TGCT, increasing to threefold in TGCT patients with a family history of the disease (Nathanson et al., 2005). However, additional TGCT susceptibility genes are likely to exist.

Animal models can provide leads to possible candidate genes that may cause disease in humans. The *Ter* mutation is a strong modifier of TGCT risk in mice. Homozygosity for *Ter* causes complete germ cell deficiency in adult males and females, regardless of the genetic background (Sakurai et al., 1995). A homozygous *Ter* mutation on the background of the 129 mouse strain is a strong modifier of spontaneous germ cell tumor susceptibility and markedly increases the incidence of TGCT in 129-Ter/Ter males (Stevens, 1973). The *Ter* gene was mapped to mouse chromosome 18, syntenic to human chromosome 5 (Asada et al., 1994), and the gene has been isolated (Youngren et al., 2005). The *Ter* phenotype is due to a single point mutation which introduces a stop codon (R178X) in the mouse orthologue (*Dnd1*) of the zebrafish dead-end (*dnd*) gene (Youngren et al., 2005). *Dnd1* is expressed in the fetal testis during the critical period when TGCTs are believed to develop in mice. The likely functional inactivation of the *Dnd1* gene in mice leads to both severe germ cell depletion and TGCT. Comparative sequence analysis of proteins showed that *Dnd1* is closely related to apobec complementation factor, which is involved in RNA editing, suggesting a similar role for the *Dnd1* protein (Matin and Nadeau, 2005).

Human *DND1* is located on chromosome band 5q31.3 and consists of four exons (NM_194249). The mouse model of germ cell tumorigenesis most closely reflects that of TGCT in children, which are believed to be clinically, genetically and epidemiologically distinct from the more common type of germ cell tumors in adolescents and adults (Looijenga and Oosterhuis, 1999). Therefore, the mouse may not represent the ideal animal

model for germ cell tumors in adolescents and adults. As *DND1* is expressed in the developing testis and there are likely to be multiple genes for TGCT susceptibility, we postulated that *DND1* is a candidate gene for TGCT. We investigated *DND1* for mutations in a series of adult TGCT patients with and without a family history of disease.

MATERIALS AND METHODS

Two patient series were used for this study. The initial “test” series consisted of cases of TGCT identified from the Royal Marsden Hospital testicular cancer clinic, Sutton Surrey, United Kingdom and from a national United Kingdom study of familial testicular cancer coordinated at the Institute of Cancer Research. The second series consisted of index testicular germ cell tumors (TGCT) cases (with and without family history) collected by the *International Testicular Cancer Linkage Consortium* (ITCLC) with additional samples included from the United Kingdom TGCT collection.

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.

Control samples were drawn from the 1958 Birth Cohort. This is an ongoing follow-up of all individuals born in Great Britain during 1 week in 1958, including a recent biomedical assessment during 2002–2004 at which blood samples and informed consent were obtained for creation of a genetic resource (www.cls.ioe.ac.uk/studies.asp?section=000100020003). More than 97% of the controls were of white ethnicity.

DNA was extracted from whole blood using standard techniques. The coding region of the human *DND1* gene was examined using 6 primer pairs (Table 1), designed from ensembl database information for the *DND1* gene (ENSG00000183403) (http://www.ensembl.org/Homo_sapiens) using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). All exons, except exon 3 (see below) of *DND1* were examined by conformation sensitive gel electrophoresis (CSGE) (Ganguly et al., 1993). Briefly, both PCR primers were labeled with adenosine - 5' (γ - ^{32}P) triphosphate by T4 polynucleotide kinase. After amplification PCR products were heated to 98°C and cooled down to 60°C for more than 30 min to allow heteroduplex formation. PCR samples were run on a CSGE gels [10% v/v ethanediol, 13.75% v/v formamide, 15% v/v acrylamide with 4 mg/ml piperazine and 1× GTB buffer (89 mM Tris, 29 mM taurine, and 0.5 mM EDTA)]. To evaluate the presence of homozygous mutations by CSGE, the amplified sample was “spiked” with unlabeled control DNA amplified for the same primer pair. PCR products from samples that showed migration shifts on CSGE were bidirectionally sequenced using BigDye terminator v3.1 sequencing kit and a 3730xl automated sequencer (Applied Biosystems, Foster City, CA).

The sequence for exon 3 was GC rich and a PCR product could not be amplified under standard PCR conditions. Each fragment of exon 3 was therefore amplified using “GC-rich PCR system” (Roche-diagnostics, Basel, Switzerland) and then sequenced in a single direction using BigDye terminator v3.1 sequencing kit and a 3730xl automated sequencer (Applied Biosystems, Foster City, CA).

The variant p.Glu86Ala was evaluated across a larger series of TGCT patients and controls by TAQMAN assay. Primers were designed by “assay by design” (Applied Biosystems, Foster City, CA). Primers and probes were as follows:

Forward primer: TGTACGAGCACCAGCTTA TCC

Reverse primer: GCCGCTGAAGGTCATCATCA

Vic labeled probe: CCTCTACGAGTTCCG

Fam labeled probe: CCTCTACGCGTTCCG

Samples were amplified using manufacturers recommended conditions and examined on a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). All samples showing the p.Glu86Ala variant by TAQMAN were bidirectionally sequenced using BigDye terminator v3.1 sequencing kit and a 3730xl automated sequencer (Applied Biosystems, Foster City, CA) to confirm the change.

RESULTS

Two hundred and sixty-three index TGCT patients were examined for point mutations, small deletions, and insertions in the entire *DND1* coding sequence by CSGE and direct sequencing. Of this series of patients, 165 had a family history and 98 cases had no family history of TGCT. Of the 165 patients with a family history, 45% were sib pairs (Table 2).

DND1 contains four exons and was examined in six fragments (Table 1). In exon 3, sequence analysis demonstrated a c. t407c change resulting in a synonymous p. His121His amino acid change. Fifty patients were homozygous c/c, 114 were heterozygote c/t and 99 patients were homozygous t/t giving a frequency of 59.3% for the “t” allele and 40.7% for the “c” allele. This SNP is recognized in dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) as rs2563333 and the allele frequencies determined from this data reflect that in dbSNP. Further, the SNP is in Hardy-Weinberg Equilibrium ($P = 0.09$). No additional coding SNPs are recorded for this gene in dbSNP and no additional SNP polymorphisms were identified in this study.

A c. a301c change was seen in single TGCT case causing an amino acid substitution of p. Glu86Ala in exon 3 (Fig. 1). The amino acid residue at this position is highly conserved through evolution and is in a known functional domain (Fig. 2). The patient (TS151-1) presented with stage 1 nonseminoma of the left testis aged 24 years. He underwent surgery and surveillance but relapsed after 1 year. Subsequently, he underwent four cycles of bleomycin, etoposide, and cisplatin (BEP) but, 4 years after diagnosis relapsed with cerebral and right pulmonary metastases and died. The patient had no documented family history of TGCT and no history of UDT. The patient had fathered two children before diagnosis of the disease. Tumor material from this case was unavailable.

To evaluate the frequency of this variant in control populations and in other TGCT patients we designed a TAQMAN assay to identify this single base change. We tested 2,066 controls (1,024 females and 1,042 males) and no instances of p. Glu86Ala were detected.

We then investigated a second series of TGCT patients, including cases from the ITCLC set (Table 2). Eight hundred and forty-two samples from index TGCT cases, 269 cases with and 573 TGCT cases without family history of TGCT were examined by TAQMAN to identify the p. Glu86Ala variant. A further 246 samples from TGCT cases were examined (111 from the ITCLC and 135 from the United Kingdom) in pedigrees from which the index case also had been examined. No additional p. Glu86Ala variants were identified in these sets.

DISCUSSION

We investigated the *DND1* gene in a series of patients with TGCT, with and without a family history of TGCT, to evaluate whether this gene contributed to TGCT susceptibility. The majority of the *DND1* gene was screened for mutations by CSGE, which does not detect

all sequence variants and large deletions or duplications are unlikely to be detected by this methodology. However the sensitivity of CSGE is high and compares favorably with other high throughput methods of mutation detection (Leung et al., 2001; Ganguly, 2002).

One silent variant was demonstrated in exon 3; it is a recognized SNP (rs2563333) in dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>). A further variant- p. Glu86Ala, was detected in a single patient of 1105 index TGCT cases and was absent in control individuals (0/2066). p. Glu86Ala is within the RNA binding domain of *DND1* and the amino acid at this position is conserved throughout evolution and is present in mouse, zebrafish and xenopus (Fig. 2). Although the variant could be a rare polymorphism, a change at such a highly conserved residue is characteristic of a disease-causing allele. Without examining larger cohorts of cases and controls, we cannot assess whether *DND1* p. Glu86Ala is disease-causing or not. However, mutations in *DND1* make, at most, a very small contribution to TGCT susceptibility.

The genetics of TGCT is complex and despite the high relative risk, susceptibility genes have yet to be identified. A recent linkage search has demonstrated that susceptibility to TGCT is not due to a single gene with major effects but rather is likely to arise through multiple genes with modest effects on risk (Crockford et al., 2006). Animal models, such as the mouse, provide novel candidates that can be evaluated in humans to determine if these contribute to TGCT susceptibility. Although mutations in *DND1* in humans are unlikely to contribute significantly to TGCT in adults and adolescents, given the similarities between the mouse model and childhood TGCT an investigation of this gene in a series of childhood TGCT patients would be warranted.

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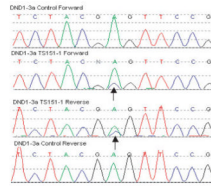


Figure 1.
The c. a301c change causing an amino acid substitution of p. Glu86Ala in exon 3 of *DND1* in index TGCT patient, TS151-1.



Figure 2.
DNA sequence and amino acid sequence of human DND1 gene. Evolutionary conservation of amino acid sequence in a variety of species.

TABLE 1PCR Primer Pairs for Analysis of *DND1* Gene

Exon	Forward primer	Reverse primer	PCR fragment size bps
Exon 1	GTGGAGGGGTAGGGAAGC	AGAGGAAAGGAAGGTTGGA	248
Exon 2	GACCCAGTACCACAGGAGA	CGGGTAGTTCGCAGTTTCTG	288
Exon 3a	ACGGGTGTAAACCCATTCA	AGCTCACACTTCTCGGTGCT	400
Exon 3b	CCTATGCCCGCTACAGCTC	AAGATCTTGTAACCTTAACAGCTATGC	390
Exon 4a	CAGGAAATGGGAAGGCTACA	CCCAGGAATCACCACTG	396
Exon 4b	GCTGTGCCAACGAATGAAG	ACCTTTGGGGGTCAGAAAGT	379

TABLE 2

TGCT Series Examined for Mutations in *DND1*

TGCT patient category	Series 1	Series 2			Overall total
		UK	ITCLC	Total	
Family history of TGCT	165	72	197	269	434
Sib Pair	74	32	68	100	174
Sib Trio	2	2	5	7	9
Half siblings	3	1	0	1	4
MZ twins	4	2	1	3	7
Father son pairs	25	17	42	59	84
Grandfather Grandson pairs	4	0	6	6	10
Cousin pairs	23	5	25	30	53
Uncle nephew pairs	18	8	15	23	41
Large pedigrees > 3 affected cases	12	4	26	30	42
Other relationship	0	1	9	10	10
Bilateral TGCT and no family history	6	18	75	93	99
TGCT with no family history	84	408	50	458	542
TGCT with a family history of UDT	8	12	10	22	30
Total	263	510	332	842	1,105

MZ, Monozygotic.

Series 1 cases were examined for the entire *DND1* gene, series 2 were examined by a TAQMAN assay for the p. Glu86Ala variant only. Only index cases are presented. An additional 246 samples were examined 111 from the ITCLC and 135 from the UK. These samples were the other affected relatives from familial TGCT pedigrees.