Stem Cell Factor Receptor (c-KIT) Codon 816 Mutations Predict Development of Bilateral Testicular Germ-Cell Tumors

Leendert H. J. Looijenga,1 Hubert de Leeuw,1 Monique van Oorschot,4 Ruud J. H. L. van Gurp,1 Hans Stoop,1 Ad J. M. Gillis,1 Carlos A. de Gouveia Brazao,2 Rob F. A. Weber,2 Wim J. Kirkels,3 Thamar van Dijk,4 Marieke von Lindern,4 Peter Valk,4 Gezcy Lajos,5 Edit Olah,6 Jahn M. Nesland,6 Sophie D. Fossà,6 and J. Wolter Oosterhuis1

1Pathology/Laboratory for Experimental Patho-Oncology, Erasmus MC, University Medical Center Rotterdam, Daniel den Hoed Cancer Center, Josephine Nefkens Institute, Rotterdam, the Netherlands; 2Andrology, 3Department of Urology, and 4Department of Hematology, Erasmus MC, Rotterdam, the Netherlands; 5Departments of Chemotherapy C and Molecular Genetics, National Institute of Oncology, Budapest, Hungary; 6Department of Pathology and Clinical Research, the Norwegian Radium Hospital, Oslo, Norway

ABSTRACT

Testicular germ-cell tumors (TGCTs) of adolescents and adults originate from intratubular germ cell neoplasia (ITGCN), which is composed of the malignant counterparts of embryonal germ cells. ITGCN cells are characterized, among others, by the presence of stem cell factor receptor c-KIT. Once established, ITGCN will always progress to invasiveness. Approximately 2.5–5% of patients with a TGCT will develop bilateral disease and require complete castration, resulting in infertility, a need for lifelong androgen replacement, and psychological stress. To date, the only way to predict a contralateral tumor is surgical biopsy of the contralateral testis to demonstrate ITGCN. We did a retrospective study of 224 unilateral and 61 proven bilateral TGCTs (from 46 patients, in three independently collected series in Europe) for the presence of activating c-KIT codon 816 mutations. A c-KIT codon 816 mutation was found in three unilateral TGCTs (1.3%), and in 57 bilateral TGCTs (93%; P < 0.0001). In the two wild-type bilateral tumors for which ITGCN was available, the preinvasive cells contained the mutation. The mutations were somatic in origin and identical in both tumors. We conclude that somatic activating codon 816 c-KIT mutations are associated with development of bilateral TGCT.

Our report the novel finding that the presence of an activating codon 816 c-KIT mutation in a TGCT is associated with development of a contralateral TGCT. An activating c-KIT codon 816 mutation, encoding an Asp816His, has been reported previously in a single TGCT (16).

INTRODUCTION

TGCTs7 of adolescents and adults, the seminomas and nonseminomas (1), are the most frequent malignancies in Caucasian males 15–40 years of age (2). Several risk factors have been reported, including family history, undescended testis, and a previous TGCT (see Ref. 3 for review). The initiating event, leading to ITGCN, also known as carcinoma in situ (4), occurs during intrauterine development (5, 6). ITGCN cells indeed show characteristics of embryonic germ cells, i.e., PGCS/gonocytes, including the presence of the stem cell factor receptor c-KIT (7) and OCT3/4 (PUS51; Ref. 8).

Between 2.5 and 5% of TGCT patients will develop contralateral disease. Approximately half of the second tumors are diagnosed within the first 5 years after the initial diagnosis. However, ~25% of patients present with the second tumor 10 years after diagnosis of the first, and the latency period can be up to 20 years (9, 10). This illustrates the need for a long follow-up of all TGCT patients. To date, no predictor for risk of bilateral TGCT is available, except an open surgical biopsy for the identification of ITGCN (11). Because of possible complications, application of this procedure is not recommended for all patients with unilateral disease (12). However, early diagnosis of contralateral ITGCN has clinical relevance. This is illustrated by the fact that the lifetime risk for TGCT is similar to the incidence of ITGCN in the general population, i.e., no spontaneous regression of ITGCN occurs (13). Moreover, 50% of patients with ITGCN in the contralateral testis will develop an invasive TGCT within 5 years after diagnosis of ITGCN (14). Development of a second invasive TGCT will, in the majority of patients, require complete castration, resulting in need for hormone supplementation and psychological burden. ITGCN can be cured by low-dose irradiation, thereby preventing the development of an invasive tumor with preservation of the hormonal function in most patients (15, for review).

In view of this, there is clinical need for a predictive noninvasive test to identify TGCT patients at risk for bilateral disease. Here we report the novel finding that the presence of an activating codon 816 c-KIT mutation in a TGCT is associated with development of a contralateral TGCT. An activating c-KIT codon 816 mutation, encoding an Asp816His, has been reported previously in a single TGCT (16).

MATERIALS AND METHODS

Sample Handling and Characterization. The fresh tumors were collected in the southwestern part of the Netherlands. Representative parts of the tumors and adjacent testicular parenchyma were snap frozen or fixed overnight in 4% formalin for paraffin embedding. Diagnosis was made according to the recommendations of the WHO (1). This was confirmed by immunohistochemistry for the presence of OCT3/4, as described previously (8).

Additionally, two independent series were included, one collected in Hungary, consisting of DNA from 18 tumors (all second tumors), and a series of 6 unilateral TGCT with matched contralateral ITGCN-containing biopsies, collected in Norway.

From multiple 3- to 5-μm-thick paraffin sections, tumor and ITGCN cells were dissected after deparaffinization with a sterile scalpel blade under direct microscopic visualization. Multiple 20-μm-thick tissue sections from frozen samples were used. The cells were microdissected with the Arcturus Pixcell II system, after which DNA was isolated (digestion with I mg/ml proteinase K).

REMS AatII PCR. A modified 3′ primer (exon 17R) was used to generate an AatII recognition site encompassing codon 816 (see Fig. 1). The presence of a mutation at this codon will result in disruption of the recognition site for the restriction endonuclease, leading to nondigestion amplified products. Amplification was performed by PCR analysis using primers exon 17F (5′- TGTACTACAGACAGCTTGCA-3′) and exon 17R (5′-TTAATTAGTT-CATTCTTGAAC-3′), which yielded a 106-bp product. Reactions were carried out in a volume of 30 μl containing 2.3 mM MgCl2, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 μM deoxynucleotide triphosphates, 0.33 μM each primer, and 1.0 unit of Taq DNA polymerase (Qiagen). For each reaction, 50 ng of input DNA was used. PCR amplification was performed on a MJ Research Ther-
mocycler with the following conditions: 95°C for 5 min of initial heat denaturation and 35 cycles of 95°C for 30 s, 45°C for 30 s, and 72°C for 30 s. Amplification products (10 μl) were digested to completion with 5 units of AarII (Fermentas) at 37°C overnight, yielding 85- and 21-bp fragments for wild-type amplification products. In contrast, in the presence of a mutation, no cleavage occurred.

The amplification products were cloned into a PCR TOPO-II vector and transformed into TOPO 10 One-Shot cells by use of the TOPO TA cloning kit (Invitrogen, Breda, The Netherlands). Inserts were sequenced (at least 10) on a Licor automated sequencer using a M13 Reverse primer.

**SNuPE.** Amplification was performed with primer set exon 17F (5’-TGTTTCTTTGACACG-3’) and exon 17R (5’-GTTTCCTTTAACACACATCTTGTGATT-3’) and yielded products 122 bp in length (for conditions, see above, with an annealing temperature of 56.5°C; see Fig. 2, left panel). Amplified fragments were purified by electrophoresis on a nondenaturing 10% polyacrylamide gel containing ethidium bromide, after which the fragments were dissected and eluted with elution buffer (containing 0.5 M ammonium acetate, 1 mM EDTA) at 37°C overnight. The SNuPE reaction (total volume, 10 μl) contained 1.0 μl of extracted PCR product, 1 pmol of reverse Y/Hr (5’-ATAATTAGAATCATTCTTGATGTT-3’) SnuPE primer for D816Y and H, and, for D816V, reverse primer D816Vr (5’-CATAATTAGAATCTTCTGATGTT-3’); 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM MgCl2; 0.75 units of Tag DNA polymerase (Qiagen); and 0.2 μl of [α-32P]dATP or dGTP (3000 Ci/mmol; Amersham). Conditions for extension were 1 cycle of 95°C for 1 min, 58°C for 30 s, and 72°C for 30 s. The samples were analyzed by electrophoresis on a 15% denaturing polyacrylamide gel and subsequent autoradiography. As controls, cloned fragments of both proven mutated and wild-type sequences were used.

**RESULTS**

To screen for codon 816 mutations, we applied a REMS PCR approach to 110 primary consecutively collected TGCTs, including seminomas and nonseminomas (Fig. 1, left panel). Four TGCTs (3.6%) demonstrated indigestible products. The presence of a mutation was confirmed by sequencing of cloned amplification products (examples in Fig. 1, right panel) and the SNuPE method (Fig. 2, left panel). Two tumors showed an Asp816Val and two an Asp816Tyr. The latter two were from patients diagnosed with bilateral disease. Both TGCTs contained the same mutation (Table 1, cases 1 and 2). Additionally, 20 proven bilateral TGCTs (for which 33 tumors were available; Table 1), were investigated as were 116 randomly picked unilateral cases, all collected in the Netherlands. Although only two of the unilateral TGCTs tested in the second screen (1.7%) showed a mutation (Asp816Val), all but two tumors (94%; see below) from the bilateral cases contained a mutation (Table 1; Fig. 2, right panel). All matched tumors showed concordant findings, although an additional mutation was found in one of the tumors in seven independent cases.

Two tumors (cases 15I and 19II) did not show a mutation (Table 1), even after microdissection of tumor cells. Adjacent testicular parenchyma containing ITGCN was available for 10 of the bilateral tumors, including the 2 lacking a mutation. The ITGCN cells were microdissected and subsequently investigated. All samples showed at least one mutation, including the two samples adjacent to the wild-type tumors, most often the same mutation that was present in the matched invasive tumor (Table 1; Fig. 2, right panel). This indicates that the genetic change is present in the preinvasive lesion but can be lost on invasive growth. In three cases, semen obtained before surgical removal of the affected testis was investigated, and the same mutation was identified as present in ITGCN (Table 1).

Peripheral blood DNA from four patients with a c-KIT mutation in the tumor (two with bilateral disease), collected before surgical removal of the affected testis, was available and investigated. None of...
The presence of a GTC (encoding a valine; V), a GAC (encoding a tyrosine; Y), or a CAC (encoding a histidine; H) instead of a GAC (encoding an aspartate; wild type; WT) is specifically detected. The SNuPE primers are indicated as dotted lines. A radioactively labeled A nucleotide (underlined) is incorporated at position 2 of codon 816 in case of a GTC sequence and at position 1 in case of a GAC sequence, whereas a radioactively labeled G is incorporated at position 1 in case of a CAC sequence. None of these primers will lead to extension of an A or G nucleotide if only wild-type amplification products are present. Right, examples of detected mutations (V, Y, and H, indicated by an arrow) are shown. Both wild-type (WT) and mutated samples are indicated. Samples, both tumor (T) and precursor cells (ITGCN), are investigated without (Total) and after microdissection (Micro). In addition, cloned amplification products (plasmid) of wild-type or mutated alleles (Vplasmid, tyrosine; Yplasmid, valine; Hplasmid, histidine), as well as H2O (−) are included as controls. The codes for the samples correspond to the codes used in Table 1. For some samples, the results obtained from DNA isolated from frozen (F) and paraffin-embedded (P) tissue are shown.

them contained the mutation (see Table 1). In addition, no mutation was found in the normal testicular parenchyma lacking ITGCN in the patients with bilateral disease. In 13 invasive TGCTs and in all ITGCN samples, the mutation was found only after microdissection (Table 1).

To confirm the association between c-KIT codon 816 mutations and bilateral TGCT, we investigated an independent series of 18 TGCT of patients with bilateral disease, for whom only DNA from the second tumor was available (see Table 2, top). Sixteen of the samples contained a mutation in codon 816 (89%). In addition, an independent series of six unilateral invasive TGCTs and matched ITGCNs of the contralateral testis (obtained by biopsy) were studied (see Table 2, bottom). Of one unilateral invasive tumor, only the ITGCN cells were available. No invasive TGCT was present in any of the biopsied testes. All primary TGCTs as well as the matched ITGCN of the same testis showed a mutation. All contralateral ITGCNs showed at least the same genetic change as the primary invasive tumor and matched ITGCN. Multiple mutations were found in four ITGCN samples, whereas a single mutation was detected in two.

**DISCUSSION**

Our data convincingly show that mutations affecting codon 816 of c-KIT are associated with development of bilateral TGCT. This was proven by investigation of three independently collected series of bilateral TGCTs. Although only a minority of unilateral TGCTs contained a codon 816 mutation (1.3%), it was present in the majority of the series of bilateral TGCTs (95, 89, and 100%, respectively; P < 0.0001, Fisher’s exact test). This finding is of relevance to understand the biology of TGCT, because the c-KIT/stem cell factor system is crucial for the proper migration and survival of PGCs (see Ref. 17 for a review). The stem cell factor is also one of the crucial components for in vitro survival of mouse and human PGCs (17), as well as human seminoma cells (18). c-KIT is a tyrosine kinase receptor of the subclass III family, for which the stem cell factor is the receptor. Binding of the ligand to the receptor results in receptor homodimerization and subsequent tyrosine kinase activity, leading to phosphorylation of various substrates. Downstream signaling pathways include proteins involved in regulation of apoptosis and cell proliferation (see Ref. 19 for a review). The codon 816 mutations result in a constitutional kinase-activated protein and phosphorylation of tyrosine residues, although with a change in the catalytic activity and substrate specificity compared with the wild-type receptor. No differences have been identified to date between the various types of codon 816 mutations regarding their biochemical properties.

This study shows that the c-KIT mutation in TGCTs is somatic in origin, in accordance to the findings of the International Testicular Cancer Linkage Consortium.8 Because of the presence of similar changes in the matched bilateral TGCTs, the findings support the model that bilateral TGCTs are monoclonal in origin. Although clinically unlikely (9, 10), the second TGCT might be a metastasis of the primary tumor to the contralateral testis. This possibility was excluded in our study by our investigation of six cases of unilateral invasive TGCT with matched contralateral ITGCN (without invasive disease), the latter identified by surgical biopsy. These findings favor the model

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that the mutation occurs during or before migration of PGCs to the genital ridge.

Of the 224 unilateral TGCTs, 4 (1.8%) showed a mutation. One of these patients developed a bilateral TGCT (Table 1, case 3) during the period of this project. The second TGCT in this patient was diagnosed 9 years after the first. To date, none of the other three patients developed a second tumor. No contralateral biopsy was done. The ages of these patients at initial diagnosis were 26, 34, and 41 years, up to 20 years, in accordance with published data (9, 20). Because of the knowledge that no spontaneous regression of ITGCN occurs (13), these patients will be kept under close examination.

At least two of the patients with a bilateral TGCT from our investigated series (Table 1, case 8; Table 2, top, case I) had an affected brother (for which the tumors were not available for analysis). This indicates that the presence of a c-KIT mutation is not restricted to sporadic TGCT, which was confirmed in the study of the International Testicular Cancer Linkage Consortium.8 That study restricted to sporadic TGCT, which was confirmed in the study of the International Testicular Cancer Linkage Consortium.8 That study showed no germline mutations in 240 pedigrees of families with at least two patients with TGCT. In addition, a significantly higher number of c-KIT mutations were found in bilateral versus unilateral TGCT. The finding that not all bilateral TGCTs in the familial context contained a mutation might be because of the familial predisposition. Familial predisposition might be an alternative mechanism to a c-KIT mutation for promotion of bilateral disease. Alternatively, it might be attributable to the fact that no ITGCN was investigated. We showed that although the invasive TGCT could lack a mutated c-KIT, the mutation is present in the precursor lesion. Further investigation could shed light on the possible role of common intrauterine etiological factors in the development of TGCT (21).

We demonstrated that a mutation of codon 816 in c-KIT is associated with development of bilateral TGCT. These kinds of mutations
contralateral ITGCN, were identified based on biopsy analysis (collected in Norway) see Hungary). In the bottom portion, the primary tumor with adjacent ITGCN, as well as stromal tumors. In contrast, this small molecule does not inhibit c-
mutations. The tyrosine kinase inhibitor STI571 (imatinib mesylate; is in contrast to the intrinsic kinase activity in the case of codon 816 receptor dimerization, leading to kinase activity on the receptor. This reported in gastrointestinal stromal tumors, do not affect exon 17 but
mutations, as mastocytosis, acute myeloid leukemia, and sinonasal natural killer/T-cell tumor (16). In addition, they have been identified in human have been found previously in a single TGCT and an ovarian germ
(15). It remains to be investigated whether specific tyrosine kinase
have been found previously in a single TGCT and an ovarian germ cell tumor (16). In addition, they have been identified in human mastocytosis, acute myeloid leukemia, and sinusonal natural killer/T-cell lymphoma (see Ref. 19 for a review). The activating mutations, as reported in gastrointestinal stromal tumors, do not affect exon 17 but do affect exons 9 or 11. These mutations result in ligand-independent receptor dimerization, leading to kinase activity on the receptor. This is in contrast to the intrinsic kinase activity in the case of codon 816 mutations. The tyrosine kinase inhibitor STI571 (imatinib mesylate; Gleevec) has been found to be effective for treating gastrointestinal stromal tumors. In contrast, this small molecule does not inhibit c-KIT containing codon 816 mutations (see Ref. 19 for a review), as found in TGCT (Ref. 16 and this study).

The activating mutations detected in TGCT likely occur during early embryonal development. Therefore, c-KIT codon 816 mutations are highly penetrant genetic changes for embryonic germ cells, leading to development of bilateral TGCT. The presence of such a mutation in a TGCT indicates a high risk for bilateral disease. Because of the occasional absence of the mutated allele in the invasive tumor, analysis of both the invasive tumor and the ITGCN is recommended. This finding could modify present management and counseling of patients with a unilateral TGCT, making open surgical biopsy for identification of contralateral ITGCN superfluous in the majority of patients with a unilateral TGCT. It could facilitate improved treatment of patients prone to develop bilateral disease, either by a watch-and-wait strategy or by use of localized low-dose gonadal irradiation, which should not affect hormonal function in the majority of patients (15). It remains to be investigated whether specific tyrosine kinase inhibitors, such as PP1 and PP2 (22), might be effective in chemoprevention of progression of contralateral ITGCN to an invasive TGCT in patients with a clinically proven unilateral TGCT with a c-KIT codon 816 mutation.

ACKNOWLEDGMENTS

We thank Elizabeth Rapley and Mike Stratton (Institute of Cancer Research, Section of Cancer Genetics, Sutton, Surrey, United Kingdom) for critical reading of the manuscript.

REFERENCES