Amplification and Overexpression of the KIT Gene Is Associated with Progression in the Seminoma Subtype of Testicular Germ Cell Tumors of Adolescents and Adults


Abstract
We have previously identified amplification at 4q12 in testicular germ cell tumors of adolescents and adults centered around the KIT gene encoding a tyrosine kinase transmembrane receptor. Analysis of primary testicular germ cell tumors totaling 190 cases revealed 21% of the seminoma subtype with an increased copy number of KIT whereas this change was rarely found in the nonseminomas. In most cases, gain of KIT did not include the immediately flanking noncoding DNA or the flanking genes KDR and PDGFRA. Increased copy number of KIT was not found in the putative precursor lesion, carcinoma in situ (CIS), adjacent to tumor with this change. KIT overexpression was found independent of gain and KIT immunostaining was stronger in selected cases with gain of KIT compared to those without. Taken together with activating mutations of KIT in exon 17 identified in 13% of seminomas, this suggests that the KIT gene product plays a role in the progression of CIS towards seminoma, the further understanding of which may lead to novel less toxic therapeutic approaches. (Cancer Res 2005; 65(18): 8085-89)

Introduction
Testicular germ cell tumors of adolescents and adults are the most common tumor in young men and are increasing in incidence (1). They can be classified into the two main histologic categories of seminoma and nonseminoma. Seminoma resembles germ cell origin, and nonseminoma is composed of neoplastic tissues exhibiting either somatic, embryonic, or extraembryonic tissues (1). Primordial germ cells or gonocytes are widely accepted as the cells of origin of testicular germ cell tumors and are believed to give rise to carcinoma in situ (CIS), a precursor lesion of both seminoma and nonseminoma. Through comparative genomic hybridization analysis of testicular germ cell tumors using a cDNA microarray, we recently identified gain in copy number of a small region at 4q12 centered around the KIT gene (2). KIT encodes a tyrosine kinase transmembrane receptor for stem cell factor, and activation through phosphorylation and dimerization is associated with ligand binding and specific mutations. Activating mutations of KIT have been identified in testicular germ cell tumors (3–5). KIT signaling has a significant role in normal germ cell development through its effects on cell survival and proliferation (6). The genes KDR and PDGFRA flank KIT. KDR encodes a receptor for vascular endothelial growth factor (VEGF), and expression of VEGF has been previously correlated with vascular density and metastasis in testicular germ cell tumors (7). PDGFRA lies distal to KIT and encodes the platelet-derived growth factor (PDGF) receptor α, which, like KIT, is a tyrosine kinase receptor protein. Activated KIT, KDR, and PDGFRA may all exert their effects through phosphatidylinositol 3-kinase and/or RAS signaling (8). The importance of KIT mutations in tumors, such as gastrointestinal stromal tumors, and the involvement of PDGFRA in the fusion protein associated with idiopathic hypereosinophilic syndrome have led to the use of small molecular inhibitors of KIT and PDGFRA kinase activity in the management of patients (9). As gain and amplification of genes may indicate their specific involvement in tumor development, we have investigated here the potential involvement of KDR, KIT, and PDGFRA in the development of testicular germ cell tumor. Through analysis of a large number of testicular germ cell tumor samples, we exclude amplification of the PDGFRA and KDR genes flanking KIT in some cases and associate amplification and increased expression of KIT with progression to invasive tumor.

Materials and Methods
Tumor samples. Testicular germ cell tumor samples were snap-frozen in liquid nitrogen directly after surgical removal. The samples analyzed were 111 seminoma, 73 nonseminoma, 6 combined tumors, 14 testicular samples with different amounts of CIS ranging from 8% to 100%,7 as well as 19 normal testis parenchyma samples. Six single samples (5 seminoma and 1 nonseminoma) were from patients with sporadic bilateral disease and two further cases of seminoma were from cases with a familial history of testicular germ cell tumor. Samples were collected through the Royal Marsden Hospital NHS Trust and in close collaboration with urologists and pathologists in Rotterdam and surrounding regions. Ethical approval and appropriate consent has been obtained for the use of samples in this study. The cell lines Tera1, Tera2, GCT27, and GCT44 were cultured as previously

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7 http://www.crukdmf.icr.ac.uk/array/array.html.
Figure 1. A. PDGFR, KIT, and KDR copy numbers in selected seminoma samples. Light shaded columns, PDGFR; black columns, KIT; dark shaded columns, KDR copy number ratio. i, examples of samples with no amplification.

B. KIT copy number data for CIS and adjacent tumor. Black columns, copy number ratio in CIS adjacent to invasive tumor. Light shaded columns, copy number ratios of the tumor.

Tumor samples. Expression data were normalized to the commercially available pooled normal testis RNA and genomic data to normal male DNA. Reactions using 2× Universal TaqMan Mastermix (Applied Biosystems) were labeled with Vic and all test sets were labeled with Fam. The copy number of KIT was calculated using linear regression analysis from an external, commercially available RNA standard curve. The subsequent relative copy numbers were determined by taking the mean value of KIT over the adjacent marker SHGC-50974.

Amplification of KIT determined by fluorescence in situ hybridization. Interphase fluorescence in situ hybridization (FISH) was done on tumor touch imprints from one tumor sample and a normal lymphocyte control as previously described (11). A probe for the centromere of KIT was investigated in the six selected paraffin-embedded testicular germ cell tumor sections. Sections were incubated at 4°C at a 1:500 dilution as previously described (12). B. KIT copy number data for CIS and adjacent tumor. Examples of samples with no amplification. A. PDGFR, KIT, and KDR copy numbers in selected seminoma samples. Black columns, copy number ratio in CIS adjacent to invasive tumor. Light shaded columns, copy number ratios of the tumor.

Quantitative-PCR analyses. Quantitative PCR and reverse transcription-PCR (RT-PCR) were done using the ABI PRISM 7700 Sequence Detection System according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA).8 Samples were studied at two centers using two different methods. For 32 seminoma, 27 nonseminoma, and 6 combined tumors, β2-microglobulin (B2M) was used as the endogenous control in expression studies and hsRBP4 at 2q21 as the endogenous control for genomic quantification as this region rarely shows loss or gain in testicular germ cell tumor samples.9 Expression data were normalized to the commercially available pooled normal testis RNA and genomic data to normal male DNA. Five nanograms of DNA or cDNA were used in 10 μL multiplex PCR reactions using 2× Universal TaqMan Mastermix (Applied Biosystems; part no. 4332042). RNA was reverse transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Primer and probe sets for the genes KIT, KDR, PDGFR, and the control B2M were purchased from Applied Biosystems (assays Hs00174029, Hs0016676, Hs00184366, and Hs99999970, respectively).8 Primers and probes were designed according to Applied Biosystems guidelines for genomic analysis of KIT, KDR, PDGFR, and HsRBP4.10 Both the B2M and HsRBP4 controls used were labeled with Vic and all test sets were labeled with Fam. The quantification of each sample was determined by averaging the results from three separate reactions.

For analysis of the remaining 79 seminoma, 46 nonseminoma, 14 CIS, and 19 normal testis samples, the expression levels were quantified relative to the hypoxanthine phosphoribosyltransferase (HPRT) housekeeping gene primers HPRT 245, 5′-CGT GGG GCT CTT TTC ACC AGC AAG-3′, and HPRT 244, 5′-AAT TAT GGA CAG GAC TGA ACG TC-3′. KIT expression was quantified using the primers c-KIT forward, 5′-CTG AAC ACG CAC CTG CGA AA-3′, and c-KIT reverse, 5′-AAG CTA CGT TGC TAT TGG GAA T-3′. The PCR reactions were done in a final volume of 25 μL containing cDNA synthesized from 30 ng total RNA, 330 nmol/L primers, and 12.5 μL SYBR green PCR Master Mix (Applied Biosystems). A dissociation curve was run at the end of the reaction for product specificity. Expression data were normalized to the average of 19 normal testis parenchyma samples. Five nanograms of tumor DNA from 79 seminoma, as well as normal reference DNA, were amplified with sequence-tagged site (STS) markers (National Center for Biotechnology Information) within and flanking the KIT gene using real-time quantitative PCR. STS primers centromeric to KIT, but distal to KDR, were SHGC-14272 (UniSTS: 174218) and G16733 (UniSTS: 15362). The STS primer telomeric to KIT, but centromeric to PDGFR, was SHGC-50974 (UniSTS: 10990). The KIT-specific STS markers used were GDB:250632 (UniSTS:156301), STS-N21003 (UniSTS: 21855), and SHGC-4-128 (UniSTS: 79238). The copy number of KIT was calculated using linear regression analysis from an external, commercially available RNA standard curve. The subsequent relative copy numbers were determined by taking the mean value of KIT over the adjacent marker SHGC-50974.

Analysis of KIT protein expression in carcinoma in situ hybridization. The genomic sequence of exon 17 of KIT was amplified from 31 seminoma and 17 nonseminoma samples by PCR resulting in a 366 bp product.11 PCR products were bidirectionally screened by direct sequence analysis on an ABI3100 sequencer using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Mutation analyses of KIT. The genomic sequence of exon 17 of KIT was amplified from 31 seminoma and 17 nonseminoma samples by PCR resulting in a 366 bp product.11 PCR products were bidirectionally screened by direct sequence analysis on an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Mutation analyses were confirmed by repeat sequencing analysis from an independently amplified reaction. Negative controls (no DNA) and normal controls (pooled normal DNA) were included in every set of amplifications and sequencing analysis.

Results and Discussion

Prompted by our previous definition of a minimum overlapping region of gain centered on the KIT gene at 4q12 through cDNA microarray comparative genomic hybridization analyses (2), we have further investigated candidate genes from this

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10 Details at http://www.crukdmficr.ac.uk/array/array.html.
11 The primers are described at http://www.crukdmficr.ac.uk/array/array.html.
region in a large series of primary tumor samples and four nonseminoma cell lines.

Quantitative-PCR analyses identified gain of two or more copies of KIT in 21% of seminoma and 9% of nonseminoma based on the generally near triploid status of this tumor type. Significantly, 9 of 11 seminoma cases with gain of copy number showed amplification of only KIT and not the KDR and PDGFRA genes which flank it (Fig. 1A). In keeping with this, analysis of a further 79 seminoma cases showed that 13% of seminoma gained KIT but not the immediately flanking genomic sequences. Interphase FISH was used to confirm KIT amplification in one case and the dispersed and doublet appearance of the signals was consistent with the presence of double minutes. The number of signals was concordant with the quantitative-PCR data (25 copies by FISH and 24 copies by quantitative PCR; Fig. 3). The genomic copy number of KIT was found to be significantly greater in seminoma than in nonseminoma (t test = 4.29, P < 0.001). The specific gain of KIT in some cases, and not the flanking genes or adjacent genomic sequences, is strong evidence for selective involvement of KIT in testicular germ cell tumor, and in seminoma in particular.

Expression analyses revealed underexpression of PDGFRA and KDR relative to normal testis in primary tumor samples (the average expression ratio for KDR was 0.65, whereas, for PDGFRA, it was 0.23 with no significant differences between the tumor subtypes). The relative level of the full-length transcript of PDGFRA was measured although a shorter 1.5 kb transcript from a promoter within intron 12 has also been detected in testicular germ cell tumors (13). The two seminoma patient samples which had amplification of KDR and PDGFRA, in addition to KIT, were determined to have relatively lower expression levels for KDR and PDGFRA than the average found in seminoma as a whole. This indicates that the amplification of KDR and PDGFRA is not associated with their overexpression. The specific lack of amplification in many cases and the general decrease in expression of KDR and full-length PDGFRA suggest that increased expression of these genes may be detrimental to these tumors.

In line with the copy number changes for KIT, KIT expression was found to be significantly different between seminoma and nonseminoma (t test = 6.51, P < 0.001; Fig. 2). This corresponded to a general increase in expression in seminoma relative to normal testis whereas there was a general decrease in expression in nonseminoma samples compared with normal testis. High expression of KIT was identified in one combined tumor and several seminoma without corresponding genomic gain. This suggests that mechanisms other than copy number gain can result in KIT overexpression. Expression of KIT 6-fold greater than that found in normal testis was determined in four malignant teratomas. Strong immunohistochemical staining for KIT has been described in the

Figure 2. A, copy number and expression ratios for KIT in seminomas (i), combined tumors (ii), nonseminomas (iii), and CIS from sample series 1 testicular germ cell tumor (iv). Light shaded columns, copy number ratio of tumor samples and cell lines; black columns, corresponding expression ratios. Arrows, samples with exon 17 activating mutations in KIT. B, log 2 expression ratio of KIT for seminomas (i), nonseminomas (ii), normal testis (iii), and CIS from sample series 2 (iv) compared with the average of the normal testis samples.
differentiated teratoma structures of nonseminoma tumors (14). The immunohistochemical staining for KIT was stronger in four seminoma cases with amplification and overexpression of KIT compared with two cases without (Fig. 3).

Gain of KIT was not found in CIS adjacent to tumor material showing this gain, implying selection during progression from CIS (Fig. 1B). This was supported by antibody staining for KIT, which in four of six testicular germ cell tumor samples investigated showed increased expression of KIT in the tumor compared with adjacent CIS (Fig. 3). Quantitative RT-PCR results show a significant difference in KIT expression between seminoma and CIS (t test = 6.65, P < 0.001) where the average expression in seminoma was 36-fold greater than in CIS. Reported immunostaining generally identifies KIT expression in CIS and seminoma but results are variable (14–16). KIT expression may be a feature of the cellular origin of CIS and seminoma (3) although the specific gain and up-regulation of KIT noted here is indicative of functional significance. An increased expression of KIT in NIH 3T3 cells has been shown to lead to transformation in a receptor density-dependent manner and could contribute to the development of testicular germ cell tumors (17).

Further evidence for the involvement of KIT comes from the finding of activating mutations, mainly in exon 17, in 1% to 25% of testicular germ cell tumors (3–5, 18). Four of 31 (13%) seminoma and none of 17 nonseminoma were found to have activating mutations in exon 17. The activating KIT mutations were two D816V, one Y823C, and one N822K. Six of the seminoma samples studied were from individual sporadic bilateral cases, one of which showed an activating mutation of KIT (N822K). This is a similar rate to that observed in a recent study by Rapley et al. (18), but lower than the 93% of bilateral cases reported in which activating codon 816 KIT mutations were suggested to be predictive of developing a tumor in the contralateral testis (4).

The pattern of expression and mutation suggests that increased expression of KIT is not necessarily required in cases with an activating mutation, and it is possible that increased expression of KIT is an alternative way these tumors activate the KIT pathway. No distinct pattern of activation of downstream targets has been determined in cases with or without activating KIT mutations and similar activated levels of Erk, AKT, mitogen-activated protein kinase, and signal transducers and activators of transcription 3 have been recently reported in almost all seminomas and nonseminomas (3, 19). It is possible that alternative receptor tyrosine kinases are also involved.

Specific amplification of KIT and increased expression associated with invasive progression may recapitulate features of KIT signaling pathways important in primordial germ cells for controlling proliferation, migration, and survival (6). The implication and further understanding of the role for KIT in the development of testicular germ cell tumors may provide a target for treatment that is less toxic than current regimens for metastatic seminoma.

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