Up-Regulation of Cyclin-dependent Kinase 4/Cyclin D2 Expression but Down-Regulation of Cyclin-dependent Kinase 2/Cyclin E in Testicular Germ Cell Tumors

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ABSTRACT

Testicular germ cell tumors (GCT) characteristically display two chromosome 12 abnormalities: the isochromosome i(12p) and concomitant deletions of the long arm. Some genes important in the control of the G1/S cell cycle checkpoint G1S, i.e., cyclin-dependent kinases 2 and 4, cyclin D2 are located on this chromosomal region. Therefore, testicular GCTs were analyzed as to the expression of CDK2, CDK4, CDK6, and the expression of their catalytic partners cyclins D1, D2 and E by semiquantitative reverse transcription-PCR. Cyclin D2, located on 12p2, was overexpressed in 69% (31 of 45) of the tumors by a mean factor of 8, including all histological subtypes. In addition, the cyclin D2 partner CDK4 was increased in 41% (21 of 51) of all tumors by a factor of 6, most strongly in embryonal carcinomas. Sixty-four percent of the seminomas and 23% of the non-seminomas had decreased expression of CDK6 by a mean factor of 5 (P = 0.0009). Statistical analysis using configural frequency analysis and regression analysis revealed that cyclin D2 and CDK4 expression were strongly correlated (r² = 0.682; P = 0.000052), whereas expression of CDK6 did not correlate with either of them (r² = 0.382; P = 0.00085). CDK2 and its catalytic partner cyclin E were down-regulated in 40% (19 of 47) and 42% (19 of 45) of the tumors, respectively, by a factor of 7 each. Western blots and immunohistochemical experiments confirmed cyclin D2 and CDK4 overrepresentation and reduced expression of cyclin E and CDK2 tumors in the few tumors under protein study. Despite its localization on 12q13, a hot spot for loss of heterozygosity in testicular GCTs (>40%), Southern blotting revealed no gross DNA alteration of the CDK2 gene. Because up-regulation of the cyclin D2/CDK4 complex and down-regulation of cyclin E/CDK2 complex were found in seminomas as well as non-seminomas in the few tumors under protein study, this finding has been confirmed by various investigators (6 – 8), its diagnostic and prognostic relevance is still under discussion (9, 10). Additional abnormalities involve deletions of the long arm of chromosome 12, particularly the 12q13 and 12q22 regions, in 40% of all histological subtypes (11). These findings suggest the localization of one or more tumor suppressor genes on 12q and the localization of relevant proto-oncogenes on 12p (12). The acquired data supports the hypothesis of a possible role for chromosome 12 in the development and progression of testicular GCTs.

An important molecular change in testicular GCTs involves the loss of mRNA and protein expression of the RB tumor-suppressor gene. We found a highly significant suppression or lack of expression of the RB gene at both mRNA and protein levels in 95% and 71%, respectively, of seminomas and non-seminomas (13). The RB protein was not detected by immunohistochemistry in undifferentiated tumors (seminomas, embryonal carcinoma and chorion carcinomas). Only differentiated cells of teratocarcinomas and mixed tumors showed some protein expression. However, using Southern blotting, no alterations were found at the DNA level (13).

The nuclear protein encoded by the RB gene is consistently expressed by all normal mammalian cells and tissues. However, a number of different tumor types (RB, bronchial carcinoma, osteosarcoma, glioma, and carcinoma of the prostate and bladder) are associated with inactivation of the RB gene by deletion, mutation and/or lack of down-regulation (14 –16). The RB protein is an essential protein in cell cycle regulation, and its function is regulated by phosphorylation. In G0 and the early G1 phase, hypophosphorylated RB is complexed with the cellular transcription factor E2F (17, 18). In late G1, a significant hyperphosphorylation of the RB protein by cyclin-dependent kinases (CDK2, CDK4, and CDK6) in complex with their catalytic partners (cyclins D1, D2, D3, and E) occurs (19 –22). As a consequence, E2F is set free and binds to promoters of genes that are involved in the early steps of the subsequent S phase, i.e., DNA polymerase α and δ, dihydrofolate reductase, thymidine kinase, cyclin E, CDK 2, c-myc, and E2F-1 itself. During the subsequent S, G2, and mitosis phases, the RB protein remains hyperphosphorylated.

Several regulators of RB are located on the regions of chromosome 12 frequently affected by aberrations in GCTs (23 –25). While human cyclin D2 gene (CCND2) is located on 12p13, the human CDK2 gene (CDK2) and CDK4 gene (CDK4) are localized on 12q13. These findings, combined with the above-mentioned observations on RB expression in GCTs, give rise to the hypothesis that the cell cycle restriction point G1/S may be entirely disrupted in testicular GCTs. The aim of this study was to test this hypothesis in primary testicular tumors...
Cyclins and CDKs in Testicular Tumors

MATERIALS AND METHODS

Tumor and adjacent normal testicular tissue was obtained by surgery, shock frozen in liquid nitrogen, and stored at −70°C. The tissue cohort included 58 GCTs and the adjacent nonmalignant testicular tissues. Histological subtype and staging was performed according to the International Histological Classification of Tumors (26) and the American Joint Committee on Cancer (27).

Isolation of Total RNA. Total cellular RNA was extracted from frozen tissues using guanidinium thiocyanate, phenol-chloroform, and CsCl density gradient centrifugation as described (28). Purified RNA was quantified by UV spectroscopy scans from 340 nm to 210 nm (UviKon 900; Kontron Instruments, Neufarm, Germany). Integrity of RNA was confirmed by denaturing PAGE.

Primers. Primers specific for cyclin E, cyclin D1, cyclin D2, cyclin D2 pseudogene, CDK2, CDK4, and CDK6 cDNA were designed using sequences published previously (24, 25, 29–35) by the primer design program Oligo, Version 4.4. Primers for cyclin D1, cyclin D2, CDK2, and CDK4 have been published previously by our laboratory (34). Primer sequences are summarized in Table 1. The human D-type cyclin genes CCND1 (cyclin D1), CCND2 (cyclin D2), and CCND3 (cyclin D3) share an average of 57% identity over the entire coding region and 78% in the cyclin region. The cyclin D2 and its pseudogene are 86% homologous. The downstream primer for cyclin D2 cDNA was therefore chosen to bind in a small region deleted in the pseudogene. All primers for RT-PCR were selected to span one or more introns to avoid amplification of residual DNA (Table 1).

Semiquantitative RT-PCR. Because our extracted RNA was used in former Northern expression studies on the metastasis suppressor genes nm23 and DCC and the c-myc oncogene (36, 37), we had not enough RNA for additional Northern blotting. Therefore, we performed semiquantitative RT-PCR with highly sensitive northern chemiluminescence detection as described previously (36). cDNA was synthesized by RT from 2 μg total RNA using oligo(dT) primer (Sigma Chemical Co., St. Louis, MO) and AMV reverse transcriptase (Promega, Madison, WI). One-tenth of the cDNA was used for amplification by PCR. Amplification of cDNAs was performed using 20-pmol primers each. DNA polymerase (1 unit; DynaZyme; Biometra, Goettingen, Germany), 50 μM dNTP mixture, and 2.5 μM digoxigenine-labeled dUTP (Roche Diagnostics, Mannheim, Germany) were used for each reaction. An initial 5-min denaturation at 95°C and 5 min annealing at the indicated temperature (Table 1) were carried out. Cycles of polymerization, denaturation, and annealing were then performed for 90 s at 72°C, 30 s at 96°C, and 45 s at the empirically determined optimal annealing temperature. The ranges of cycle numbers yielding amplification were determined for each gene in preliminary experiments. The housekeeping genes GAPDH and/or β-actin were coamplified as an internal control. To compensate for low expression of some cell cycle regulators, addition of control gene primers to the PCR reaction was delayed by a number of cycles (compare Table 1). PCR products were separated on a 1.6% agarose gel and transferred to a nylon membrane (Hybond; Amersham, Buckinghamshire, England) according to the manufacturer’s protocol. Chemiluminescence was detected on enhanced chemiluminescence-Hyperfilm (Amersham). Signals were quantified by video scanning densitometry (One D-SCAN; Scanalytics, Billerica, Madison, WI) and standardized for β-actin expression in each matched normal testicular tissue.

One set of experiments was performed with GAPDH as the internal control. However, because of the localization of the GAPDH gene on chromosome 12p, GAPDH expression was additionally standardized to β-actin expression in each tumor/normal pair. Overexpression in tumor was defined as expression >2-fold of the corresponding normal tissue; normal as between 0.5-fold and 2.0-fold; and decreased expression as <0.5-fold, after standardization to the β-actin expression ratios of tumor and normal tissue.

Southern Blot Analysis. Genomic DNA (10 μg) was digested with 8 units of TaqI restriction enzyme (Life Technologies, Inc., Karlsruhe, Germany), resolved on an 0.8% agarose gel, and transferred to a nylon membrane (Hybond; Amersham, Buckinghamshire, UK) according to standard protocols (28). Prehybridization and hybridization were performed in Dig-Hybridization buffer (Roche Diagnostics, Mannheim, Germany) at 42°C. Fifty ng of the 778-bp CDK2 probe (pCDK2), representing nucleotide numbers 166–943 (kindly provided by Guido Reifenberger, Department of Neuropathology, University of Dueseldorf, Dueseldorf, Germany) was digoxigenine-labeled by oligonucleotide random priming (38) and used for hybridization. The final wash was carried out in 0.5× SSC-0.1% SDS at 68°C.

Western Blot Analysis. Tissues were pulverized using mortar and pestle on dry ice. Tissue powder was added directly to ice-cold lysis buffer containing 25 mM Tris-HCI (pH 7.4), 50 mM sodium chloride, 0.5% sodium deoxycholic acid, 2% NP40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, and 50 μg/ml leupeptin and incubated for 15 min on ice. Lysates were boiled for 10 min, passed through a 25-gauge needle three times, and centrifuged at 14,000 × g for 15 min. Protein concentration from the supernatant was quantified by using bicinchoninic acid assay (Pierce Biochemicals, Rockford, IL). Five to 25 μg of total cellular proteins were mixed with one-fifth volume of 5× SDS loading buffer [250 mM Tris-HCI (pH 6.8), 10% SDS, 50% glycerol, and 0.001% bromphenol blue] and boiled for 5 min. Together with a prestained protein marker, these samples were separated by 10% SDS-PAGE and transferred by semidyblotting to Immobilon membranes (Millipore, Bedford). Membranes were probed with mouse monoclonal antibodies against cyclin D1 (clone HD 11; dilution: 1:200; Oncogene Research Products, Boston, MA), cyclin E (clone HE12; dilution: 1:1000), CDK6 (clone B10; dilution: 1:1000), goat polyclonal antibodies against cyclin D2 (dilution: 1:1000), CDK4 (dilution: 1:1000), and CDK2 (dilution: 1:500). All antibodies, with the exception of cyclin D1 antibody, were purchased from Santa Cruz Biotechnology, Heidelberg, Germany. Detection was performed using antimouse and antigoat antibodies conjugated with horseradish peroxidase and the enhanced chemiluminescence system (Amersham Life Sciences).

Immunohistochemistry for Cyclin D2, Cyclin E, CDK4, and CDK2. Paraffin-embedded, formalin-fixed tissue sections (5 μm) were deparaffinized in xylol and rehydrated. Sections were treated with 0.1% H2O2 in methanol for 30 min. Antigen retrieval was achieved by microwave treatment in 1.8 mM citrate buffer (pH 6) for 10 min (cyclin E) or treatment with 1 mg/ml Pronase E (Merck, Darmstadt, Germany) in PBS for 1 h (cycline D2), or treatment with 0.0125% trypsin solution (Roche, Mannheim, Germany; pH 7.6), for 1 h (CDK 4 and CDK 2). Nonspecific binding was blocked by incubation with rabbit serum (DAKO, Glostrup, Denmark) for cyclin D2, CDK 2, and CDK 4, and horse serum for cyclin E (Vector, Burlingame), for 30 min. Primary antibodies used for immunohistochemistry were the same as those for Western Blotting. Primary antibodies were diluted 1:500, 1:100, 1:500, and 1:200 for

Table 1. RT-PCR primers and experimental conditions used for analyzing mRNA expression of G1/S cell cycle regulators

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Annealing temperature</th>
<th>No. of cycles</th>
<th>Product length</th>
<th>Source</th>
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<tr>
<td>CDK 2</td>
<td>5’GCTTCTGCTGATTTCTCCATC-3’</td>
<td>5’GTCGAGAGAAATAAGGAT-3’</td>
<td>57°C</td>
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<td>(34)</td>
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<td>CDK 4</td>
<td>5’AAGCTATGTCGATCCAGCT-3’</td>
<td>5’TGGTGGTTGTTGCTATGAG-3’</td>
<td>59°C</td>
<td>18</td>
<td>464 bp</td>
<td>(34)</td>
</tr>
<tr>
<td>CDK 6</td>
<td>5’GAGACTCTCCACCCTCAGAT-3’</td>
<td>5’CCACCCAGCATATCC-3’</td>
<td>62°C</td>
<td>23</td>
<td>499 bp</td>
<td>This paper</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>5’GAGAACCCTACATTGGACAGCT-3’</td>
<td>5’TCTTGAGCCCTTCCTCCG-3’</td>
<td>65°C</td>
<td>22</td>
<td>484 bp</td>
<td>(34)</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>5’TCTAGATTCGCTGCCCTC-3’</td>
<td>5’TTAGGTCGCTGACACAACA-3’</td>
<td>65°C</td>
<td>19</td>
<td>247 bp</td>
<td>(34)</td>
</tr>
<tr>
<td>Cyclin D2 pseudo</td>
<td>5’TACGAGAACATGCAAGACAGAC-3’</td>
<td>5’CCACCTGCAAGAACTTCT-3’</td>
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<td>29</td>
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<tr>
<td>Cyclin E</td>
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<td>5’TCCAGAGACATTGCTGAGATGACGGG-3’</td>
<td>61°C</td>
<td>15</td>
<td>661 bp</td>
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<tr>
<td>β-actin</td>
<td>5’TGCAGGCGGCGACACCCACAGCTCTGCT-3’</td>
<td>5’TACCTGACCCACAGCTGTCCT-3’</td>
<td>59°C</td>
<td>15</td>
<td>379 bp</td>
<td>(35)</td>
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</table>

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antihuman cyclin E, antihuman cyclin D2, antihuman CDK 2, and antihuman CDK 4, respectively. Incubation was performed overnight at 4°C. Biotinylated secondary antibodies (Vector) were applied for 1 h with subsequent incubation with horseradish peroxidase-conjugated streptavidin (Vector) for 1 h. Visualization was achieved by incubation with diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and counterstaining with Mayer’s hematoxiline (Merck, Darmstadt, Germany).

Statistical Analysis. All statistical tests were carried out using the SAS program, Version 6.12, and SPSS, Version 9.0. The program provided in Ref. 39 was used for CFA. The significance of differences of expression between different histological subtypes and tumor stages was analyzed using Fisher’s exact test and the $\chi^2$ test (including odds ratios and confidence intervals). Statistical evaluation of the correlation between mRNA expression of each pair of the regulators was performed by linear and quadratic regression analysis. Possible relations between the five cell cycle regulators were analyzed using the WHO Histological Classification of Tumors (26) and the American Joint Committee on Cancer (27).

RESULTS

In the first sets of experiments, we used the housekeeping gene GAPDH as internal standard. However, because of chromosomal localization of GAPDH on 12p, GAPDH was found overexpressed in testicular tumors. Therefore, we standardized all expression results from all tumors, including those with GAPDH, to $\beta$-actin expression. GAPDH was increased by a mean factor of 3.5 in 55% of all tumors (Fig. 1). There was no specific correlation of GAPDH expression to tumor stage or histological subtype.

Expression of Cyclins. Cyclin D2 was expressed in normal testicular tissues and was found to be overexpressed in 69% (31 of 45) of all testicular GCTs by a mean factor of 7.9 with various histologies. This overexpression affected both seminomas and non-seminomas. Strong overexpression of cyclin D2 was found in 80% (17 of 21) of the localized tumors and in 58% (14 of 24) of the tumors with lymph node and/or distant metastases (Table 2). This difference was not statistically significant ($\chi^2, P = 0.102$; Fisher, $P = 0.121$; odds ratio, 3.036) but allowed this interpretation in form of a trend (Table 3). We excluded expression of the cyclin D2 pseudogene (31) as the cause for overexpression by a subset of tumors with specific primers resulting in a longer PCR product during amplification of cyclin D2 pseudogene (data not shown). The cyclin D2 pseudogene was expressed neither in tumor nor in normal testicular tissue of ten patients. Analysis of an additional 10 normal and tumor tissues of different histologies revealed only minute cyclin D1 mRNA expression, although we used three more amplification cycles than in cyclin D2 PCR. In contrast, cyclin D1 expression was found with teratocarcinoma cell lines TERA-1 and TERA-2 (see Fig. 2), urothelial cancer cell lines VMCub I, VMCub III, and TCC Sup, and prostate cancer cell lines DU145 and LNCaP using the same amplification profile (published previously by our group; see Ref. 34).

Expression of cyclin E mRNA was decreased in 42% (19 of 45) of GCTs by a mean factor of 6.8, predominantly in seminomas (in 58% versus 36.4% in non-seminomas). Interestingly, most of the tumors with lowered cyclin E expression were localized tumors (11 of 21; 52%), whereas only 29% (7 of 24) of the tumors with lymph node
Analysis confirmed this finding as a trend (Table 3, and/or distant metastasis showed this change (Table 2). Statistical analysis supported this finding (Table 3), revealing a trend and/or significance in Fisher’s exact test and the χ² test (Table 3).

Expression of CDKs. CDK4 expression was increased in 41% (21 of 51) of all tumors. This increase was more frequent (16 of 34; 47%) in non-seminomas than in pure seminomas (5 of 17; 33%) and was most prevalent in embryonal carcinomas (5 of 7; 71%; Table 2). However, the average increase in expression was more pronounced in the seminomas (factor 7.9) than in non-seminomas (factor 5.5). Although there was an increase in percentage of CDK4 overexpression with tumor stage, there was no significance in Fischer’s exact test and the χ² test (Table 3).

In contrast, CDK2 mRNA expression was strongly decreased in 40% (19 of 47) of the GCTs (Fig. 3). This decrease affected both seminomas and non-seminomas, but was much more pronounced (9.2-fold) in the pure seminomas than in the non-seminomas (4.5-fold). Interestingly, only 2 of 12 (17%) teratocarcinomas had decreased CDK2 expression. There was an inverse relationship to tumor stage, with the highest frequency (13 of 25; 52%) and the strongest decrease (9.2-fold) in localized tumors (Table 2). This finding is supported by statistical analysis (Table 3), revealing a trend (P = 0.085; odds ratio 2.89) for this relationship.

Most of the tumors had normal CDK6 expression, but 36% (16 of 44) showed a down-regulation of the gene by a mean factor of 4.7. Nine of 14 (64%) seminomas, but only 7 of 30 (23%) of the non-seminomas were affected (Table 2). This difference was highly statistically significant (P = 0.009; odds ratio = 5.9; Table 3). There was no correlation to tumor stage.

Analysis of the CDK2 Gene. To analyze a loss of the gene as a potential cause for a decreased CDK2 expression, DNA from tissue pairs of patients with decreased CDK2 expression was studied by Southern hybridization (Fig. 4). However, the data revealed no differences in gene copy number or in gross structural alteration of the CDK2 gene in these patients, with the exception of tumor 27, which seems to have a decreased number of copies or a loss of the gene.

Configural and Correlation Analysis. To find possible relations between any combination of the five cell cycle regulators CFA including the ascending and descending ISA was performed. ISA was used in a first calculation for any pair of the regulators CDK2, CDK4, CDK6, cyclin D2, and cyclin E and in a second calculation for any higher order of interactions between them. The first calculation revealed only one strict relation, i.e., between CDK4 and cyclin D2 (Table 4). However, additional interactions were found for the following triplets: CDK4, CDK2, cyclin D2; CDK4, cyclin D2, cyclin E; CDK4, cyclin D2, CDK6. Higher combinations (one versus three, two versus two, and one versus four) showed no significant interaction. All results were proven by the asymptotic hypergeometric version of CFA (Table 4). Thus a strong correlation between two regulators is only given for CDK4 and cyclin D2, which is the same result as that from regression-analysis (Fig. 5a). This finding is valid for high levels of expression of CDK4 and cyclin D2 and for low levels of expression of both, too. To get significance in correlated expression, at least three regulators have to be considered for all other relations (Table 4; high expressions in CDK4, CDK2, and cyclin D2; and high expressions of CDK4, cyclin E, and cyclin D2). The interaction between CDK4, cyclin D2, and CDK6 found with ISA could not be confirmed with CFA and thus had to be rejected.

As expected from the results of the ISA, there was a linear correlation between cyclin D2 and CDK4 (r² = 0.585; Fig. 5a, left) which was even improved in a quadratic model (r² = 0.682; Fig. 5a, right). For all other combinations, no strict correlation (r² > 0.5) was obtained, in agreement with the result from ISA. At least three factors have to be considered for a strict correlation. A slight correlation between CDK4 and CDK6 (Fig. 5b; r² = 0.2652 in the linear model, and r² = 0.381 in the quadratic model) was found using regression-analysis.

Analysis of Protein Expressions. To investigate whether mRNA expression data were mirrored by protein expression data, we performed preliminary Western blotting analyses and immunohistochemistry from a subset of tumors (Figs. 6 and 7). However, we had residual tissues and paraffin-embedded tissue blocks from only a few patients, which had been previously analyzed by RT-PCR for mRNA expression. By comparing the tumor/normal ratios of each cell cycle

<table>
<thead>
<tr>
<th>Method</th>
<th>Regulator</th>
<th>P</th>
<th>Adjusted α</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISA</td>
<td>CDK 4, cyclin D2</td>
<td>0.000052</td>
<td>0.00000052</td>
</tr>
<tr>
<td>ISA</td>
<td>CDK 4 × (CDK 2, cyclin D2)</td>
<td>0.001574</td>
<td>0.0001786</td>
</tr>
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<td>ISA</td>
<td>CDK 4 × (cyclin E, cyclin D2)</td>
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<td>ISA</td>
<td>CDK 4 × (CDK 6, cyclin D2)</td>
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<td>0.0001786</td>
</tr>
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<tr>
<td>CFA</td>
<td>CDK 4 (low), cyclin D2 (low)</td>
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<td>0.0005000</td>
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<td>CFA</td>
<td>CDK 4 (high), cyclin D2 (high)</td>
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<td>0.0000125</td>
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<td>CFA</td>
<td>CDK 4 (high), cyclin E (high), cyclin D2 (high)</td>
<td>0.000001</td>
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</table>
regulator as determined by video scanning densitometry of Western and RT-PCR bands, mRNA expression data for cyclin D2, cyclin E, and CDK6 could be clearly confirmed (Table 5). In addition, CDK4 overexpression as well as CDK2 down-regulation was found in this subset of tumors, although the level of expression ratios (tumor: normal) for proteins and mRNA were not the same for every tumor. Contrary to negligible mRNA expression (see Fig. 2), cyclin D1 protein expression was pretty high in tumor and normal testicular tissues (Fig. 5).

To confirm further cyclin D2 and CDK4 overexpression and cyclin E and CDK2 down-regulation in testicular tumors, we performed immunohistochemical analysis for these G1-S cell cycle regulators (Fig. 7). In normal testicular tissues, mainly later stages of spermatogenesis (spermatids) demonstrated strong nuclear cyclin D2 staining, whereas Sertoli cells and the early stages of spermatogenesis were negative (Fig. 7a). In contrast, seminoma cells (Fig. 7b) had strong nuclear and cytoplasmatic cyclin D2 staining. CDK4 was not detected in normal testicular tissue, neither in germ cells nor in Leydig or Sertoli cells. However, nuclear CDK4 staining was detected in smooth muscle cells of blood vessels in the same section (Fig. 7c). In contrast with normal tissues, testicular tumors had heterogeneous CDK4 expression, with very high levels of CDK4 in tumors with epithelial differentiation and weaker expression in tumor stroma (Fig. 7d). Cyclin E protein was strongly expressed in seminiferous tubules of normal testicular tissue in basal layers with spermatogonia (Fig. 7e). Embryonal carcinomas as shown in Fig. 7f displayed completely negative tumor cells, whereas infiltrating lymphocytes and capillaries (arrows) stained positive for cyclin E in the same section. CDK2 expression was found mainly in Sertoli cells of normal testicular tissues (Fig. 7g). The seminoma shown in Fig. 7h was completely negative, with the exception of endothelial cells of blood vessels within the tumor.

**DISCUSSION**

Because of the chromosomal localization of several G1-S restriction point cell cycles regulators on chromosome 12, which is typically altered in testicular GCTs, we analyzed the expressions of several G1-S regulator molecules involved in the regulation of RB function. The mRNA of the cyclin D2 gene, located on 12p13, was strongly overexpressed by an average factor of 7.9 in 69% of all tumors throughout all histological subtypes. Although we did not do any studies on cyclin D2 gene amplification, with our tumors used for expression studies, our result on cyclin D2 overexpression is in accordance with the presence of isochromosome i(12p) in >80% of the testicular GCTs published by others (6–8), and we believe that this overrepresentation of 12p may be the cause of cyclin D2 overexpression. However, the housekeeping gene GAPDH, which is located on the same band on chromosome 12, was overexpressed by a mean factor of only 3.3. One recent triple-color FISH study of 12p amplifications in 49 testicular GCTs using 12p band-specific painting probes demonstrated the amplification of the three bands 12p11.2, p12, and p13 in all tumors with copy numbers varying from 4–11 (41). Eighty-four percent of the tumors displayed one or three isochromosomes 12, and many had multiple copies of parts of 12p elsewhere in the genome. The authors concluded that several genes located in different parts on the distal region of 12p (12p12 and/or 12p13) are involved in the pathogenesis of testicular tumors. Therefore, the difference between cyclin D2 and GAPDH mRNA overexpression may be caused by the fact that two or more regions of 12p13 may be amplified to different degrees.

The first evidence suggesting cyclin D2 to be involved in the pathogenesis of GCTs came from Sicinski et al. (42), who developed cyclinD2 knockout mice. Although widely expressed during normal mouse embryo development, cyclin D2 knockout animals were phenotypically indistinguishable from their wild-type littermates, possibly because of the expression of other cyclin D types in most tissues. However, mutant females were infertile because of an incapacity to release oocytes, whereas mutant males displayed hypoplastic testis. In addition, these authors found very high mRNA expression levels in cell lines from yolk sac tumors and an increase in the copy number of
the cyclin D2 gene in 15 of 19 cell lines tested. Houldsworth et al. (43) found a marked variation of the cyclin D2 mRNA expression level between six cell lines derived from testicular GCTs, but no correlation to the copy number. The protein was overexpressed to different extents in the individual cell lines, with inverse correlation to the status of differentiation. Although normal germ cells contained no protein, seminomas with no evidence of differentiation displayed the highest protein expression. The expression patterns of teratomas correlated with the specific pathway of differentiation. Although we found cyclin D2 expression in the later stages of spermatogenesis (spermatids), these findings were confirmed by our immunohistological study (see Fig. 7, a and b) on these few tumors under study; this was not the case in our mRNA expression study, where different tumor types showed similar frequencies and extents of overexpression (Table 3). However, this is in accord with an analysis of the amplification rate of the 12p region by FISH (41), which revealed no

### Table 5 Comparison of protein expression ratios (tumor:normal) of patient samples shown in Western blots of Fig. 5 versus their mRNA expression as determined by RT-PCR

Quantification of protein and mRNA expression was done by laser scanning densitometry. mRNA expression of cyclin D1 was too low for quantification and is indicated as n.d. (not detected).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tumor ID 13</th>
<th>Tumor ID 41</th>
<th>Tumor ID 78</th>
<th>Tumor ID 100</th>
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<tbody>
<tr>
<td></td>
<td>Protein</td>
<td>mRNA</td>
<td>Protein</td>
<td>mRNA</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>2.1</td>
<td>4.1</td>
<td>0.8</td>
<td>1.1</td>
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<tr>
<td>CDK 4</td>
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<td>9.9</td>
<td>5.0</td>
<td>1.5</td>
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<td>0.3</td>
<td>0.8</td>
<td>0.1</td>
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<tr>
<td>CDK2</td>
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<td>0.4</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>0.7</td>
<td>n.d.</td>
<td>1.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>CDK 6</td>
<td>0.6</td>
<td>0.2</td>
<td>0.8</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*Fig. 7.* Immunohistochemical analysis of the expression of cyclin D2 (A and B), CDK4 (C and D), cyclin E (E and F), and CDK2 proteins (G and H) in normal testicular tissues with seminiferous tubules (left; A, C, E, and F) and testicular GCTs (right; B, D, F, and H). Tumors in B and H are seminomas, whereas the tumor in D and F represent the mixed tumor subtype and embryonal carcinomas, respectively. Magnification is ×400 in each section with the exception of C, where a magnification of ×250 was used. The arrows indicate positive staining of smooth muscle cells in a vein in C, of lymphocytes and capillaries in F, and of blood vessel cells in H.
correlation with the histological tumor type. The finding of cyclin D2 overexpression in seminomas as well as in localized tumors suggests that this occurs early in the tumorigenesis of testicular GCTs.

In a subset of nine testicular tumors and adjacent normal testicular tissue, we did not find any significant cyclin D1 mRNA expression. These results fit very well to immunohistochemical data from Bartkova et al. (44), who showed that cyclin D1 protein expression was absent in normal germ cells, seminomas, and embryonal carcinomas. They detected moderate to low cyclin D1 expression in some differentiated compounds of teratomas. Interestingly, this group found variable positivity for cyclin D3 in invasive testicular tumors as well as a correlation of cyclin D3 with differentiation, whereas normal germ cells again were negative. However, the role for cyclin D3 in promoting S-phase entry and initiation or maintenance of differentiation has to be figured out in the future.

CDK4 and CDK6 in complex with cyclin D2 act to drive the cell cycle. Because the 12q13 region where the CDK4 gene is located is often deleted in testicular GCT, a decreased expression of this gene would be expected. However, we found a CDK4 overexpression by an average factor of 6 in 41% of the tumors correlating with cyclin D2 overexpression. Although this overexpression fits to the postulated function of CDK4 as an oncogene described for glioblastomas (45) and osteosarcomas (46, 47), our mRNA expression findings are in contrast with protein expression data of six GCT cell lines, where only a little variation of CDK4 protein expression was found (43). More consistent with our data, cyclin D2 protein was in complex with CDK4 protein in these cell lines. ISA, CFA, and regression analysis of our data revealed a strict correlation between CDK4 and cyclin D2 mRNA expression in testicular tumors.

Until now there are only a few tumor subsets with known alterations of the CDK6 gene. In gliomas, an amplification-associated and an amplification-independent increase of CDK6 protein was identified (48). According to CDK6 function in phosphorylation of pRB together with cyclin D2/CDK4, an overexpression of the gene was expected. However, CDK6 overexpression was found in only 16% of the tumors. Most of the tumors had normal CDK6 expression, and 33% showed a decreased mRNA expression, particularly seminomas with high significance (64%: \( \chi^2, P = 0.009; \) odds ratio, 5.9). Houldsworth et al. (43) have also reported a decreased CDK6 protein expression in two of six cell lines and demonstrated that CDK6 was in complex with cyclin D2/CDK4. Although regression analysis of our data revealed only a weak correlated expression of CDK4 and CDK6, a highly significant interaction was revealed by ISA for the CDK4, CDK6, and cyclin D2 triple. (Table 4). However, CFA could not confirm the correlated expression of the three cell cycle regulators all together.

Loss of heterozygosity studies have revealed deletions of the 12q13 and 12q22 regions in >40% of testicular GCTs (8, 12). Several genes with relevance to tumorigenesis, including the CDK2 gene, are located in this chromosomal region, although CDK2, like CDK4, is already known to act more as an oncogene than as a tumor suppressor gene. In colorectal carcinoma, for example, the CDK2 gene is amplified, and CDK2 activity is increased (49, 50). Surprisingly, with respect to CDK2 function during G1-S cell cycle regulation, but in concordance with the previous loss of heterozygosity findings in 40% of testicular GCTs (12), we found a lowered CDK2 expression by a mean factor of 7.2 in 40% of seminomas and non-seminomas. We excluded major alterations of the CDK2 gene as the cause for decreased CDK2 expression in these tumors by Southern blots. Smaller deletions of the CDK2 gene or a transcriptional regulation of CDK2 may be the reason for CDK2 down-regulation. Our data revealed a trend for an inverse correlation with tumor stage, with the highest percentages and factors in localized tumors, indicating an early event in the tumorigenesis of testicular GCTs.

The cofactor for CDK2 action in RB phosphorylation, cyclin E recently has been suspected to be involved in tumorigenesis of breast carcinomas. For these tumors, cyclin E overexpression is suggested as a prognostic marker (51). In excellent correlation with the expression data for CDK2, we found a down-regulation of cyclin E in 42% of the testicular GCTs in both seminomas and non-seminomas. The trend for an inverse correlation to tumor stage was found to nearly the same percentages with cyclin E and CDK2. Our preliminary immunohistochemical studies confirm the decreased cyclin E expression results on the protein level, too (Fig. 7, e and f). However, decreased cyclin E protein expression was observed to higher frequencies in seminomas (85%; 23 of 27) than in non-seminomas (39%; 9 of 23). The finding of down-regulation of the cyclin E/CDK2 complex in testicular tumors is unprecedented in any tumor and is quite surprising, inasmuch as both proteins were expected to be necessary for rapid cell cycle progression. The reason for down-regulation of both regulators in testicular tumors is unclear but may be a consequence of the decreased expression of RB (13), because cyclin E transcription is positively regulated by E2F and RB protein (29).

In conclusion, up-regulation of the cyclin D2/CDK4 complex and the down-regulation of cyclin E/CDK2 complex as well as down-regulation of CDK6 in seminomas seem to be frequent and early events during tumorigenesis of testicular GCTs. In particular, overexpression of CDK4 and cyclin D2, the correlated expression of both mRNAs, and the complex formation of both proteins in GCT cell lines suggest the concerted oncogenic actions of both in these tumors. CyclinD2/CDK4 complexes may act as promoters of the cell cycle in testicular tumors despite the lack of RB. It has been shown for cyclin D1/CDK4 and cyclin E/CDK2 complexes that their overexpression can accelerate S-phase entry and promote cell cycle progression independently of RB/E2F (52), presumably by acting upon down-stream targets that are rate-limiting for S-phase entry. Interestingly, this bypass of the G1 arrest is a feature of the cyclin D1/CDK4 complex but not of cyclin D1 alone. Alternatively, overexpression of cyclin D1/CDK4 in testicular GCTs may reflect a nonphysiological loss of the specificity of the CDK4. The additional down-regulation of cyclinE/CDK2 in testicular GCTs may not have any influence on the oncogenic process, and overexpression of cyclin D2 and CDK4 may be sufficient to initiate the transforming action in testicular germ cells. The unusual type of the deregulation of the G1-S checkpoint seems to be the key event for the development of testicular GCTs.

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Up-Regulation of Cyclin-dependent Kinase 4/Cyclin D2 Expression but Down-Regulation of Cyclin-dependent Kinase 2/Cyclin E in Testicular Germ Cell Tumors

Bettina A. Schmidt, Achim Rose, Christine Steinhoff, et al.


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