Aberrant Expression of Cyclin D2 Is an Early Event in Human Male Germ Cell Tumorigenesis¹

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Abstract

Human male germ cell tumors (GCTs) arise in the spermatocytic lineage, and subsets display embryonal-like differentiation. Virtually all GCTs exhibit multiple copies of the short arm of chromosome 12, even in carcinoma in situ/intratubular germ cell neoplasia, the earliest recognizable neoplastic lesion of germ cells. Among the candidate amplified genes mapped to 12p, expression of the cyclin D2 gene (CCND2) was deregulated in a panel of GCT cell lines, with the relative level of steady-state mRNA and protein inversely correlated with the pattern of differentiation characteristic of the cell line. GCT cell lines with a more differentiated phenotype, as indicated by an immunophenotypic analysis, displayed lower cyclin D2 expression with a concurrent increase in expression of the cell cycle inhibitor p21. In the GCT cell lines in which cyclin D2 was highly expressed, cyclin D2 was in complex with its expected catalytic partners (Cdk4 and Cdk6). Whereas no detectable cyclin D2 expression was evident in normal human germ cells, cyclin D2 was expressed in the abnormal germ cells of all carcinoma in situ/intratubular germ cell neoplasia lesions studied. In GCT specimens that displayed no evidence of differentiation (seminoma) or primitive differentiation (embryonal carcinoma), cyclin D2 expression was detected. However, in tumor specimens with certain patterns of differentiation (teratoma and yolk sac tumor), expression was down- or up-regulated depending on the pattern. Our data suggest that aberrant cyclin D2 expression is an early event in germ cell tumorigenesis.

Introduction

GCTs³ in the human male provide a unique system wherein both transforming and differentiating events can be studied (1). The tumors arise in meiotic germ cells and comprise two main histological groups: seminomas (which resemble primordial germ cells in appearance) and nonseminomas. Nonseminomas exhibit various patterns of embryonal-like differentiation: primitive (embryonal carcinoma), extra-embryonic (yolk sac tumor and choriocarcinoma), and embryonic (teratoma; Ref. 1). Conventional and molecular cytogenetic analysis of a large number of tumors has shown that virtually all GCTs exhibit multiple copies of the short arm of chromosome 12 (12p) as one or more copies of an isochromosome of 12p in > 80% of cases and as tandemly repeated segments of 12p embedded in marker chromosomes in the remaining cases (2). 12p amplification has been demonstrated in CIS/ITGCN, the earliest recognizable stage of the tumor, indicating that it is one of the earliest genetic changes, if not the earliest genetic change, associated with malignant transformation of male germ cells (3). We have taken a candidate gene approach in an attempt to identify the relevant or so-called driver gene on 12p, the higher copy number and presumed aberrant expression of which may play an early role in germ cell tumorigenesis.

Among the genes mapped to 12p, we considered CCND2, FGFB, TEL, KIP1, KRAS2, and PTHLH to be candidates by virtue of their roles in normal cell cycling and proliferation. We report here that CCND2, which encodes cyclin D2, a member of the D-type cyclins, is deregulated in a panel of male GCT cell lines and aberrantly expressed in all CIS/ITGCN lesions studied. With tumor differentiation, the level of cyclin D2 expression correlated with the pattern of differentiation in both cell lines and tumors. It has recently been suggested that overexpression of the D-type cyclins, loss of p16, or loss or inactivation of pRB are means by which control of the cell cycle can be disrupted, leading to loss of genomic integrity and ultimately tumorigenesis (4–6). The possibility that early aberrant expression of cyclin D2 associated with 12p amplification in GCTs plays an early role in male germ cell tumorigenesis is discussed.

Results

12p Candidate Genes. By quantitative Southern blotting, we determined the copy numbers of six candidate genes mapped along chromosome 12p (CCND2, FGFB, and TEL at 12p13; KIP1 and KRAS2 at 12p12; and PTHLH at 12p11) in a panel of 13 male GCT-derived cell lines (data not shown). A 2–9-fold amplification of each gene was observed. Northern analysis of the steady-state mRNA levels in seven of these cell lines revealed that FGFB, TEL, and PTHLH were either not expressed or expressed at barely detectable levels (data not shown). Hybridization of multiple tissue Northern

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³ The abbreviations used are: GCT, germ cell tumor; CIS/ITGCN, carcinoma in situ/intratubular germ cell neoplasia; pRB, retinoblastoma gene product; NSE, neuron-specific enolase.
blots with these probes gave expected sizes and tissue distributions (data not shown). Although all three remaining candidates were expressed at variable levels, CCND2 was the most abundantly expressed relative to β-actin (Fig. 1a). Marked variation in the CCND2 mRNA levels was observed between cell lines and did not appear to correlate with the respective copy number (Fig. 1a). Elevated CCND2 mRNA expression was also noted in GCT biopsy specimens compared with adjacent normal testis4 and was reported to be expressed in two GCT cell lines (7). We therefore considered CCND2 to be the best candidate for

4 David Hogg, personal communication.

the 12p driver gene due to its abundant expression. In addition, the related cyclin D1 gene (CCND1) has been shown to be amplified or rearranged in a variety of tumor types leading to overexpression (4, 8).

Abundance, Subcellular Localization, and Complexing of Cyclin D2. The steady-state levels of cyclin D2 in six GCT cell lines was assessed by immunoblotting, and it was evident that the relative abundance between the cell lines, on the whole, reflected the mRNA levels (Fig. 1, a and b). This is in contrast with the normal fibroblast cell line (T89.28), in which abundant mRNA was detected, with little or no cyclin D2 evident. The GCT cell lines that displayed little or no cyclin D2 (228A and 240A) expressed relatively more cyclin D1 than did the other four lines, whereas cyclin D3 was
expressed only in the GCT cell line 240A (Fig. 1b). Immunocytochemical analysis of cyclin D2 expression in the cell line 2102E-R indicated strong dappled nuclear staining in approximately 10% of cells (Fig. 1c), with the remaining cells exhibiting a much weaker nuclear signal. This finding is consistent with a previous description of the subcellular distribution of cyclin D2 in cultured cells (9).

Because the D-type cyclins are thought to regulate the G1 phase transition by complexing with their catalytic partners cyclin-dependent kinases (Cdk4 and Cdk6) and phosphorylating pRB (5, 10, 11), we assayed for the levels of these proteins (Fig. 1b). Between the cell lines, little variation in the levels of Cdk4 was observed. The levels of Cdk6, however, were found to be much reduced in 228A and 833K-E (Fig. 1b). In the case of 833K-E, Cdk6 was detected by immunoprecipitation/immunoblotting assays (data not shown). Immunoprecipitation of cell lysates with a cyclin D2 antibody revealed that in all the cyclin D2-expressing cell lines Cdk4 was complexed with cyclin D2 (Fig. 1d). In 218A and 2102E-R, Cdk6 was also co-immunoprecipitated with cyclin D2. Hence, cyclin D2 in these GCT cell lines complexes with its expected catalytic partners. Cyclin D2-Cdk4/Cdk6 complexes were immunoprecipitated from GCT cell lines 169A, 218A, and 2102E-R with the cyclin D2 antibody and were found to exhibit kinase activity toward a fragment of pRb that could be inhibited upon preincubation with the cognate peptide (data not shown).

Recently, loss/inactivation of pRB or p16 have been implicated in the same pathway toward tumorigenesis as overexpression of a D-type cyclin (5, 6, 12–14). Although p16 (CDKN2) expression was detected in the GCT cell lines either by immunoblotting or immunoprecipitation/immunoblotting assays (data not shown), its abundance was much reduced compared to HeLa in which pRB is inactivated (12). Southern blotting indicated that CDKN2 is not homozygously deleted in any of these cell lines (data not shown). All six GCT cell lines abundantly expressed a normal-sized pRB protein, in contrast with the retinoblastoma cell line Y-79, in which pRB was virtually absent (Fig. 2a). Between the GCT cell lines, the relative level of hypophosphorylated to hyperphosphorylated pRB forms was variable (Fig. 2a). The two cell lines with little or no expression of cyclin D2 (228A and 240A; Fig. 1b) displayed a greater proportion of the hypophosphorylated pRB forms than did the other four cell lines, despite the fact that all the cell lysates were prepared from asynchronously growing cell cultures. Marked expression of p21 (a negative regulator of G1 progression by virtue of quaternary complexing with a cyclin, Cdk, and proliferating cell nuclear antigen) (6), was observed in these same two cell lines (Fig. 2b). Little or no expression of p21 was detected in the GCT cell lines expressing high levels of cyclin D2 (169A, 218A, 2102E-R, and 833K-E). Because in some cell lines and tissues increased p21 expression has been reported to be associated with a more differentiated cellular phenotype (6, 15, 16), an immunophenotypic analysis was performed on the six GCT cell lines to assess the differentiation phenotype. Cell lines 228A and 240A displayed a relatively higher level of expression of vimentin (a marker associated with a mesenchymal pathway of differentiation) and relatively lower level of cytokeratin (an epithelial cell marker), which are associated with a more differentiated phenotype (Fig. 2b; Ref. 17). In contrast, the other GCT cell lines gave a pattern of marker expression associated with a less differentiated or undifferentiated phenotype. The cell line 228A also expressed NSE, a marker, the expression of which is consistent with a neuronal pathway of differentiation often observed in GCT specimens (1). Thus, GCT cell lines displaying a relatively undifferentiated phenotype expressed cyclin D2 in significant levels, whereas more differentiated cell lines down-regulated
cyclin D2 with a concurrent increase in expression of p21 and greater proportion of hypophosphorylated forms of pRb.

**Cyclin D2 Expression in Normal Testis and GCT Specimens.** Immunohistochemical analysis of normal human testis revealed no detectable expression of cyclin D2 in germ cells (Fig. 3a), similar to that described in murine germ cells (18). In contrast, the majority of aberrant germ cells in ten CIS/ITGCN specimens studied to date displayed cyclin D2 expression (Fig. 3b). Although the staining was predominantly cytoplasmic, nuclear staining was observed in 2-5% of cells, a slightly lower percentage than for cultured cells (Fig. 1c). No cyclin D1 expression was detected in normal or abnormal germ cells (data not shown). Seminomas (GCTs that display no evidence of differentiation) expressed cyclin D2 (Fig. 4a), whereas embryonal carcinomas that display primitive postzygotic differentiation patterns exhibited a more focal pattern of expression of cyclin D2, with predominantly nuclear staining (Fig. 4b). GCT specimens with embryonic patterns of differentiation (teratoma) exhibited varying levels of expression of cyclin D2 that appeared to correlate with a particular pathway of differentiation: smooth muscle and glandular epithelium retained expression (Fig. 4c), whereas no expression was detected in differentiated epidermis and cartilage (data not shown). In GCTs that display extra-embryonic patterns of differentiation (yolk sac tumors), cyclin D2 was also expressed in a focal manner (Fig. 4d).

**Discussion**

We undertook a candidate gene approach to identify the relevant gene on the short arm of chromosome 12 that, in higher copy number in virtually all male GCTs, may play an early role in male germ cell tumorigenesis. Although all six candidate genes mapped along 12p were found to be amplified in a panel of GCT cell lines, we considered the cyclin D2 gene (CCND2) to be the best candidate due to its abundant expression. Marked variation in the relative steady-state levels of cyclin D2 mRNA and protein was observed between the cell lines and did not appear to correlate with the respective CCND2 copy number. However, an inverse correlation was indicated between the level of cyclin D2 expression and the differentiation phenotype of an individual cell line. GCT cell lines that expressed a pattern of immunophenotypic markers associated with a more differentiated cellular phenotype exhibited little or no cyclin D2 expression, with marked expression of p21 and a relatively higher proportion of pRb in hypophosphorylated forms. Immunohistochemical analysis of GCT specimens confirmed the observation that cyclin D2 is aberrantly expressed in the abnormal germ cells of the first detectable lesion in the testis (CIS/ITGCN) and in GCTs that show no evidence of differentiation (seminomas) or primitive postzygotic patterns of differentiation (embryonal carcinomas). The levels of cyclin D2 were subsequently found to vary depending on the particular pathway of differentiation. In some cases, the patterns of cyclin D2 expression concurred with levels previously noted in normal tissues and in some normal cell lines by immunoblotting (9, 12). These results indicated that aberrant expression of cyclin D2 associated with 12p amplification is an early event in male germ cell tumorigenesis.

Recent evidence in other tumor systems has indicated that abrogation of the G1-S phase checkpoint of the mitotic cycle by either overexpression of the D-type cyclins (predominantly cyclin D1), loss of p16, or loss of pRb may mediate a common pathway leading to loss of genomic integrity and ultimately tumorigenesis (4-6). Cyclin D1 overexpression has been documented in a variety of tumor systems as a result of CCND1 amplification as in breast, esophageal, and squamous cell carcinomas (19-21) or through chromosomal translocation as in centrocytic B cell lymphomas (22, 23) and inversion as in parathyroid adenomas (24). Unlike CCND1, there are only few reports of amplification and overexpression of CCND2 in human tumors and derived cell lines (7, 9, 25-27). However, the murine cyclin D2 gene has been determined to be the site of integration of a murine leukemia virus (v-in-1) in a mouse T-cell leukemia leading to overexpression of the gene (28). Hence, this is the first report of the consistent role of aberrant expression of cyclin D2 in human...
tumorigenesis. Because in the earliest GCT lesion in the testis, aberrant expression of cyclin D2 has been demonstrated in all lesions studied to date, it is possible that early aberrant cyclin D2 expression may effect the reentry of a differentiating germ cell into cycle, leading to genomic instability. Our observations of the different levels of expression of cyclin D2 in tumor lesions and derived cell lines displaying different differentiation potential are also consistent with a role for cyclin D2 early in tumorogenesis. Other transforming genetic events may subsequently alleviate the need for elevated cyclin D2 to drive cell cycle progression. Such events may include loss or inactivation of tumor suppressor genes such as DCC and of a putative tumor suppressor gene at 12q22 that are both reported to occur at relatively high frequency in this tumor system (29, 30).

One of the unique features of GCTs is the array of different differentiation patterns observed within specimens (1). Subsequently, the cell lines derived from GCTs have the capacity to display pluripotentiality (31, 32) or relatively more differentiated phenotypes (32). Two cell lines in the series used in this study displayed relatively more differentiated phenotypes (with respect to cellular immunophenotype and expression of cell cycle-related proteins), implying either derivation from a tumor cell with a similar phenotype, or that during development of the cell line, the tumor cells had undergone a differentiation process as has been described for certain pluripotent GCT cell lines (31, 32), although not to a terminal stage. Our studies have indicated that in tumor cells displaying either no evidence of differentiation (CIS/ITGCN and seminomas), or primitive postzygotic differentiation (embryonal carcinomas), cyclin D2 is expressed. However, once tumor cells initiate a differentiation process, cyclin D2 was found to be either down- or up-regulated depending on the lineage. It would be of interest to determine if the patterns of expression detected in the various tumor components were similar to those detected in their normal counterparts. Although the role of cyclin D2 in normal tissue development has yet to be described, significant cyclin D2 expression has been reported in stimulated T lymphocytes (9, 33), cultures of smooth muscle cells (12), and primary cultures and cell lines primarily of epithelial origin (9, 12), such as breast ductal cells, bronchial cells, and keratinocytes. Studies using pluripotential GCT cell lines that have the capacity to differentiate in vitro in response to agents such as all-trans-retinoic acid (31, 34) and hexamethylene bisacetamide (35) into specific lineages may elucidate mechanisms by which the cyclin D2 gene is regulated during cellular differentiation. Recent evidence has implicated a role for cyclin D1 in the normal development of the retina and in steroid-induced mammary tissue proliferation during pregnancy, as revealed in the mouse with the cyclin D1 gene homozygously deleted (36). Although homozygous deletion of the murine cyclin D2 gene may indicate a role for cyclin D2 in normal embryonic development, the results we report in
this study imply an early role for cyclin D2 in human male germ cell tumorigenesis.

Materials and Methods

Tumors, Cells, and Culture Conditions. The GCT cell lines 169A, 218A, 228A, and 240A were derived from tumor specimens obtained during surgery of patients treated at the Memorial Sloan-Kettering Cancer Center as described previously (29). The derived cell lines were tested for the presence of extra copies of 12p as described (37). The cell lines 2102E-R, 833K-E, and 577 M-F were provided by D. Bronson (Minneapolis, MN), and the colon carcinoma cell line (CCCL-17) was provided by S. Jhanwar (Memorial Sloan-Kettering Cancer Center, New York, NY). The GCT cell lines, T89.28 (normal fibroblast cell line), and HeLa were maintained in DMEM supplemented with 15% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin and streptomycin (Life Technologies, Inc.). The cell lines Y-79 (retinoblastoma cell line) and CCCL-17 were maintained in RPMI 1640 supplemented as described above, with the addition of insulin transferrin sodium selenite (Sigma Chemical Co.) for the CCCL-17 line. The GCT cell lines were grown as monolayers on flasks or dishes pre-treated with T89.28 conditioned growth medium.

Cyclin and Northern Hybridizations. DNA was extracted according to standard procedures (38), and total RNA was extracted from the cell lines at 70–80% confluency after plating using an RNaseq isolation kit (Promega). Northern and Southern hybridizations were performed and quantitated as described elsewhere (29). The filters were stripped and reprobed when necessary, and IG/JH and β-actin hybridization signals were used for normalization in the Southern and Northern hybridizations, respectively. Y. Xiong (University of North Carolina, Chapel Hill, NC), L. Suva (Beth Israel Hospital, Boston, MA), F. Coulier (INSERM, Marseille, France), D. G. Gilliland (Harvard Medical School, Boston, MA), J. Massague (Memorial Sloan-Kettering Cancer Center), R. Weinberg (Whitehead Institute, Boston, MA), J. Ravetch (Memorial Sloan-Kettering Cancer Center), and D. Beach (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY) kindly provided the CCND2, FHHL2, PGF6, TEL, KIP1, Kras2, Ig/JH, and CDK2 probes, respectively.

Protein Analysis. Cell lysates were prepared as described (39) from asynchronously growing cell cultures at 70–80% confluence after plating [asynchroney was assessed by standard flow cytometric analysis (38)]. The suspension culture Y-79 cells were collected at 24 h postseeding. For immunoprecipitation assays, protein A-Sepharose beads (Pharmacia) were precoated with rabbit antihuman polyclonal cyclin D2 antibody (%; C-17, Santa Cruz Biotechnology) in either the presence or the absence of the cognate peptide, prior to incubation with clarified cell lysate (850–950 µg of protein). Total cell lysates containing 100 µg of protein and proteins collected on the beads were electrophoretically separated in 10% SDS-polyacrylamide gels (6% for PRB and transferred to BA-S 83 membrane (Schleicher and Schuell) prior to immunoblotting according to standard procedures (38). The primary antibodies used, including cyclin D2, were cyclin D1 (HD11, mouse IgG1 antihuman monoclonal antibody), cyclin D3 (1886–11, rat IgG3, antihuman monoclonal antibody), CDk4 (C-22, rabbit antihuman polyclonal antibody), CDk6 (C-21, rabbit antihuman polyclonal antibody; all purchased from Santa Cruz Biotechnology); PRB (310T, mouse IgG, antihuman monoclonal antibody; QED, Biscience, Inc.), p21 (rabbit antihuman polyclonal antisera) (Pharmingen); and for detection, horseradish peroxidase-conjugated goat antirabbit, antimouse, or antirabbit antibodies (Santa Cruz Biotechnology) were used followed by enhanced chemiluminescence (ECL, Amersham Corp.). In the case of immunoprecipitation followed by immunoblotting, horseradish peroxidase-conjugated Protein A (Sigma Chemical Co.) was used as the secondary antibody.

Immunocytochemistry and Immunohistochemistry. For immunocytochemical analysis, cells were grown on chamber slides and fixed in 2% paraformaldehyde plus 0.1% Triton X-100 prior to antibody incubation (%), either with or without the cognate peptide (38). Following reaction with a biotinylated goat antirabbit antibody (Vector Laboratories), the cells were incubated in fluorescein-labeled avidin (Oncor) for detection purposes and counterstained with 4,6-diamino-2-phenylindole. Immunofluorescent signals and images were captured as described previously (40). Immunocytophenotyping was performed on 5-µm sections of formalin-fixed paraffin-embedded cells as described previously (17) using a standard biotin-streptavidin-peroxidase procedure with the following antibodies: mouse antiwive vimentin monoclonal (Dako Corp.), mouse antihuman cytokeratin CAM 5.2 monoclonal (Becton Dickinson), mouse antihuman NSE monoclonal (Dako Corp.), and mouse antihuman CD-30 monoclonal (Dako Corp.). The relative intensity of staining was semiquantitated because all incubations were performed at one time, and the percentages of cells displaying staining were estimated.

Immunohistochemistry was performed on 5-µm sections of formalin-fixed paraffin-embedded normal testis or tumor specimens according to standard techniques (38). For the cyclin D2 antibody (C-17, Santa Cruz Biotechnology), the optimal digestion conditions were determined on sections of normal human ovaries, in which cyclin D2 is expressed in the granulosa cells. Briefly, the sections were baked at 60°C for 1 h, deparaffinized, and microwaved at 560 MW in citric acid prior to incubation with the appropriate suppressor serum. The primary antibody (%8a) was incubated overnight. After incubation with a biotinylated goat antirabbit secondary antibody (Vector Laboratories), detection was performed using a standard streptavidin-peroxidase procedure. The tissues were counterstained with modified Harris hematoxylin (Fisher). No staining was observed upon preincubation of the primary antibody with the cognate peptide.

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