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Aberrant platelet-derived growth factor α-receptor transcript as a diagnostic marker for early human germ cell tumors of the adult testis

(carcinoma *in situ*/early detection/alternative splicing/alternative promoter use)

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ABSTRACT **Testicular germ cell tumors are the most common form of cancer in young adult males. They result from a derangement of primordial germ cells, and they grow out from a noninvasive carcinoma-*in-situ* precursor. Since carcinoma *in situ* can readily be cured by low-dose irradiation, there is a great incentive for non- or minimally invasive methods for detection of carcinoma *in situ*. We have recently shown that human Tera-2 embryonal carcinoma cells, obtained from a nonseminomatous testicular germ cell tumor, show alternative splicing and alternative promoter use of the platelet-derived growth factor α-receptor gene, giving rise to a unique 1.5-kb transcript. In this study we have set up a reverse transcriptase–polymerase chain reaction strategy for characterization of the various transcripts for this receptor. Using this technique, we show that a panel of 18 seminomas and 11 nonseminomatous testicular germ cell tumors all express the 1.5-kb transcript. In addition, a panel of 27 samples of testis parenchyma with established carcinoma *in situ* were all found to be positive for the 1.5-kb transcript, while parenchyma lacking carcinoma *in situ*, placenta, and control semen were all negative. These data show that the 1.5-kb platelet-derived growth factor α-receptor transcript can be used as a highly selective marker for detection of early stages of human testicular germ cell tumors.**

Testicular germ cell tumors (TGCTs) of adolescents and adults form the most common cancer in men between 20 and 45 years of age (1, 2). They can be subdivided into seminomas (SEs), composed of the neoplastic counterparts of primordial germ cells, and nonseminomatous TGCTs (NSs). NS can contain a variety of cell types in various states of differentiation and may include mature and immature teratoma (TE), yolk sac tumor (YS), and choriocarcinoma (CH), in addition to a population of undifferentiated stem cells, known as embryonal carcinoma (EC) cells (3). Various cell lines of human NS have been established, including the Tera-2 EC line, which can be induced to differentiate *in vitro* into a variety of nontumorigenic mature cell types upon treatment with retinoic acid (RA) (4).

Carcinoma *in situ* (CIS) is the precursor of all TGCTs, and it is believed to originate from a derangement of primordial germ cells in early life (5, 6). The total incidence of CIS in the Caucasian male population is 0.6%, and epidemiological evidence suggests that CIS, once established, will sooner or later always progress into an invasive TGCT (7). The overall cure rate of TGCTs is relatively high, but cure requires orchidectomy, mostly in combination with cisplatin treatment and/or irradiation, and still some 20% of the patients die of their disease. In contrast, CIS can readily be cured by low-dose irradiation (8), and therefore early detection could result in significant reduction of the number of patients requiring extensive treatment and an improved overall cure rate. Presently, CIS can be identified only morphologically and/or (immuno-)histochemically after surgical biopsy. CIS-specific markers would provide a major contribution to early detection of this disease by allowing analysis of samples obtained by non- or minimally invasive methods, including collection of semen and cytological aspirates (9).

The platelet-derived growth factor (PDGF) α-receptor belongs to the tyrosine kinase family of growth factor receptors, and it binds all isoforms of PDGF (10). The PDGF α-receptor gene is located on human chromosome 4 and is structurally related to the PDGF β-receptor gene and the c-*kit* and c-*fms* protooncogenes (11). The PDGF α-receptor is expressed during early mouse embryogenesis and upon further differentiation in mesodermal and glial cells (11, 12). Mice with the so-called Patch mutation lack the PDGF α-receptor gene, resulting in recessive embryonic lethality characterized by neuronal and mesodermal disorders (12). Cells expressing the PDGF α-receptor show a characteristic 6.4-kb transcript, which contains all information for the full-length receptor. We have recently shown (13) that Tera-2 EC cells show expression of the PDGF α-receptor gene with two aberrant transcripts of 5.0 kb and 1.5 kb instead. cDNA cloning combined with genomic analysis showed that the 1.5-kb transcript results from a combination of alternative promoter use and alternative splicing of the PDGF α-receptor gene. Upon cellular differentiation induced by RA, the 5.0-kb and 1.5-kb transcripts disappeared and cells expressed the normal 6.4-kb transcript instead (13).

In the present study we have set up a polymerase chain reaction (PCR)-based strategy to detect the various PDGF α-receptor transcripts in Tera-2 cells. First, we have found evidence for an additional 3.0-kb transcript in differentiated Tera-2 cells, potentially encoding a dominant-negative receptor variant. Second, we have shown that the 1.5-kb transcript is expressed not only in Tera-2 EC cells but also in a panel of SE-, NS-, and CIS-containing parenchyma samples, while no expression is observed in placenta, testis parenchyma without CIS, and normal semen. The observed selectivity, in combination with the high sensitivity of the PCR assay, makes the

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Abbreviations: CH, choriocarcinoma; CIS, carcinoma *in situ*; EC, embryonal carcinoma; HPRT, hypoxanthine phosphoribosyltransferase; NS, nonseminomatous testicular germ cell tumor; PDGF, platelet-derived growth factor; RA, retinoic acid; RT-PCR, reverse transcriptase–PCR; SE, seminoma; TCCE, teratocarcinoma cryptic exon; TE, teratoma; TGCT, testicular germ cell tumor; YS, yolk sac tumor.

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1.5-kb PDGF α-receptor transcript an ideal marker for early diagnosis of CIS.

MATERIALS AND METHODS

Cell Cultures. Tera-2 clone 13 cells were cultured and were induced to differentiate by treatment with 5 μM RA as described (13). Polyadenylated RNA was isolated from both differentiated and undifferentiated Tera-2 cells by using oligodeoxythymidylate-cellulose affinity chromatography, subjected to 1% agarose gel electrophoresis in formamide, and transferred to nitrocellulose, according to standard protocols (14).

Tissue Samples. Unilateral orchidectomy specimens suspected for the presence of a TGCT, collected at the operation theater of collaborating hospitals, were divided into two representative portions, one of which was snap frozen in liquid nitrogen, the other of which was fixed in 4% buffered Formalin and embedded in paraffin. Tumors were classified according to the recommendations of the World Health Organization (15), as described previously by Oosterhuis *et al.* (16).

For isolation of total RNA from the tumors, a series of 10–15 thick sections (thickness 4 μm) was cut from a representative frozen sample. The first and last sections were fixed for 5 min at room temperature in 100% acetone and stained with hematoxylin and eosin for histological examination. Since this protocol is unreliable for identification of CIS, this component was identified by histological staining for alkaline phosphatase, as follows. Fixed tissue sections were incubated for 10–20 min in the dark in 0.2 M Tris·HCl (pH 8.0), containing naphthol AS-MX phosphate at 0.8 mg/ml, 10% (vol/vol) *N,N*-dimethylformamide, and fast red TR at 1.1 mg/ml (all three chemicals from Sigma). Sections were counterstained with hematoxylin, washed with tap water, and subsequently embedded in 50% glycerol and 7% gelatin (Merck).

The remaining tissue sections were used for RNA isolation with the RNA-STAT-60 kit (Tel-Test, Friendswood, TX) according to the manufacturer's recommendations. All RNAs were stored at −80°C in 70% ethanol until use.

Reverse Transcriptase (RT)-PCR of PDGF α-Receptor Transcripts. For detection of PDGF α-receptor transcripts, the following PCR oligonucleotide primers were used: exon 12-specific primer (5′-GACCCGATGCAGCTGCCTTA-3′; nucleotides 1864–1883 in ref. 17), intron 12-specific primer (5′-TGCAGAAAGCTGAGGAGGCGTCTGG-3′; nucleotides 152–176 in ref. 13), exon 19-specific primer (5′-CACGGGCAGAAAGGTACTGCCT-3′; nucleotides 2684–2705 in ref. 17), and teratocarcinoma cryptic exon-specific primer (5′-GACTCAGGTTCCTCTGACATCTCG-3′; nucleotides 770–793 in ref. 13). Primers 243 and 244 (18) of the human hypoxanthine phosphoribosyltransferase (HPRT) gene were used for RNA quality control.

For RT-PCR analysis, cDNA synthesis on 0.5 μg of tumor-derived RNA was performed as described by Van Gurp *et al.* (19). After initial denaturation for 4 min at 94°C, subsequently either 32 or 42 cycles were carried out consisting of 1 min of denaturation at 94°C, 1 min of annealing at 65°C, and 2 min of extension at 72°C. The reaction vials were then incubated for 7 min at 72°C and stored at 4°C. If necessary, a booster PCR was carried out after 32 cycles by transferring one-tenth of the PCR volume (5 μl) to a vial containing fresh enzyme, nucleotides, and primers, after which another 20 PCR cycles were carried out. The same protocol was used for HPRT expression analysis using 32 cycles, with the exception that annealing was carried out at 62°C. PCR products (5 μl) were analyzed on 1.5% agarose gels in Tris/borate/EDTA (TBE; Gibco/BRL) by ethidium bromide staining and UV exposure. When necessary, DNA was transferred to Hybond N^+ nylon filters (Amersham), and hybridized with PDGF α-receptor-specific ^{32}P-labeled oligonucleotides (14).

RESULTS

PDGF α-Receptor Transcripts in Tera-2 Cells. We have previously shown by Northern blot analysis that undifferentiated Tera-2 EC cells express two PDGF α-receptor transcripts of 1.5 kb and 5.0 kb, while RA-differentiated cells (Tera-2 RA) express only a single 6.4-kb transcript. The 1.5-kb transcript has been shown to initiate within intron 12 of the PDGF α-receptor gene, and it contains exons 13–16, followed by a so-called teratocarcinoma cryptic exon (TCCE) which contains a functional polyadenylylation signal (13). The size of the 5.0-kb transcript suggested that it might result from transcription initiation at the same site within intron 12, but in combination with normal splicing to downstream exons. To investigate whether alternative promoter use and alternative splicing of

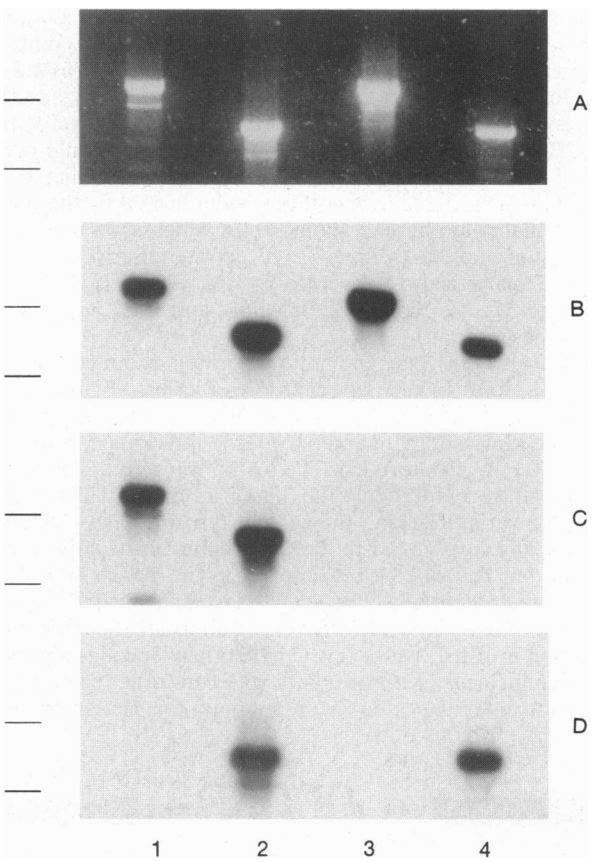

FIG. 1. Characterization of PDGF α-receptor transcripts in Tera-2 cells by RT-PCR analysis of products. Lane 1, Tera-2 EC mRNA and intron 12-specific primer in combination with exon 19-specific primer (expected size of product from 5.0-kb transcript: 850 bp). Lane 2, Tera-2 EC mRNA and intron 12-specific primer in combination with TCCE-specific primer (expected size of product from 1.5-kb transcript: 641 bp). Lane 3, Tera-2 RA mRNA and exon 12-specific primer in combination with exon 19-specific primer (expected size of product from 6.4-kb transcript: 841 bp). Lane 4, Tera-2 RA mRNA and exon 12-specific primer in combination with TCCE-specific primer (expected size of product from 3.0-kb transcript: 632 bp). Besides direct staining on gels with ethidium bromide (*A*), blots were hybridized with the following ^{32}P-labeled oligonucleotides: (*B*) Exon 16-specific primer (5′-GGACATGAAGCAGGCTGATACT-3′; nucleotides 2324–2345 in ref. 17 and 621–642 in ref. 13). (*C*) Intron 12-specific primer downstream from primer used for PCR (5′-CCAGACGCCTCCTCAGCT-3′; nucleotides 159–176 in ref. 13). (*D*) Primer specific for the exon 16-TCCE transition (5′-CCAGCCCAGCCTAACATAGA-3′; nucleotides 739–758 in ref. 13). Bars on the left represent 861-bp and 480-bp size markers derived from pKUN digested with *Taq* I.

the PDGF α-receptor gene are indeed independent phenomena, we set up an RT-PCR strategy for detection of the different PDGF α-receptor transcripts, using an exon 12-specific primer, an intron 12-specific primer, a primer downstream of exon 16 (presumably in exon 19), and a TCCE-specific oligonucleotide (sequences are given in *Materials and Methods*).

Fig. 1 shows that by using combinations of the above primers four different RT-PCR products can be detected in Tera-2 cells, depending on their differentiation state, which all hybridize with an exon 16-specific probe for the PDGF α-receptor. In Tera-2 EC cells PCR products were obtained only when the intron 12-specific primer was used, in combination with either the TCCE-specific primer or the exon 19-specific primer. These two products correspond to the 1.5-kb transcript and a 5.0-kb transcript with the above characteristics, respectively (see Fig. 2). The present characterization of the 5.0-kb transcript agrees with previous observations that this transcript does not hybridize with a TCCE-specific probe on Northern blots (13). In Tera-2 RA cells PCR products were obtained only when the exon 12-specific primer was used. In combination with the exon 19-specific primer, evidence was found for the full-length 6.4-kb transcript. An additional transcript, however, so far unknown, was found in combination with a TCCE-specific primer. The predicted transcript would correspond to exons 1–16 followed by TCCE, with a calculated size of 3.0 kb (Fig. 2). This hypothesis is confirmed by the observation that some human glioma cells, which show significant expression of a 3.0-kb PDGF α-receptor transcript by Northern blot analysis, also show hybridization with a TCCE-specific probe (S.M., M. Nistér, G. B. Afink, and E.J.J.v.Z., unpublished observation).

Fig. 2 also illustrates the putative protein products encoded by these four PDGF α-receptor transcripts. Exons 13–16, which are present in all four transcripts, encode the first tyrosine kinase domain and part of the interkinase domain of the PDGF α-receptor (13). The 6.4-kb transcript encodes the full-length receptor, while the 3.0-kb transcript will encode a receptor variant lacking the second tyrosine kinase domain, structurally very similar to that of a dominant-negative receptor mutant described elsewhere (20). The 1.5-kb and 5.0-kb transcripts potentially encode cytoplasmic truncated forms of the PDGF α-receptor. Moreover, an alternative open reading frame in intron 12 and exon 13 potentially encodes a peptide of 66 amino acids, with no homology to any other protein (13). Thus far no evidence has been obtained for protein products

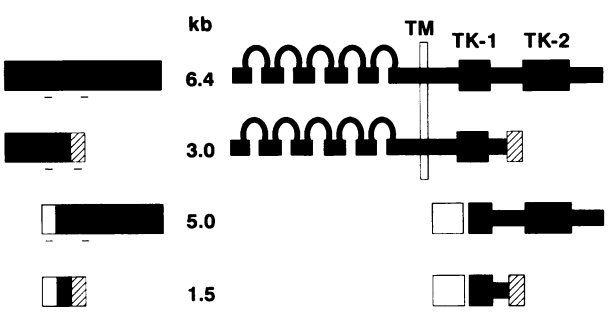

FIG. 2. Survey of human PDGF α-receptor transcripts and putative protein products. (*Left*) Organization of PDGF α-receptor transcripts, with corresponding size (kb). Black areas indicate regions corresponding to the full-length receptor sequence, gray areas indicate intron 12 sequences, and hatched areas, TCCE sequences. Lines indicate the location of the PCR primers used for individual detection of these transcripts. (*Right*) Putative protein products corresponding to the respective transcripts. Gray areas are derived from intron 12 and exon 12 sequences (alternative reading frame), and hatched areas are from TCCE. TM stands for transmembrane region, and TK-1 and TK-2 for tyrosine kinase domains. Looped structures indicate immunoglobulin-like domains.

of any of the alternative PDGF α-receptor transcripts described here.

Expression of 1.5-kb PDGF α-Receptor Transcript in TGCTs. In view of our observation that the 1.5-kb PDGF α-receptor transcript is expressed in undifferentiated but not in differentiated Tera-2 EC cells, we addressed the question of whether this transcript is also expressed in TGCTs. When the above RT-PCR analysis based on an intron 12-specific primer in combination with a TCCE-specific primer was used, all SE and NS tested were found to be positive for the 1.5-kb transcript, as shown if Table 1. All control samples, including placenta, testis parenchyma of control individuals, and control semen, were found to be negative. In addition, testis parenchyma next to a TGCT was found to be negative when devoid of both CIS and invasive malignant components. The expression levels observed were expressed as a function of the

Table 1. Expression of 1.5-kb PDGF α-receptor transcript in TCGTs

Code	Histology	Expression level*
2	SE	++
3	SE	+++
5	SE	+++
6	SE	+++
7	SE	++
8	SE	++
9	SE	++
10	SE	++
12	SE	++
13	SE	++
14	SE	++
15	SE	++
16	SE	++
49	SE	+++
50	SE	++
54	SE	+++
56	SE	++
57	SE	++
19	NS (YS, TE)	++
20	NS (YS, TE)	++
23	NS (YS, TE)	+/−
24	NS (CH, TE)	+
26	NS (EC)	+++
27	NS (TE)	+
28	NS (TE)	+
51	NS (TE, YS)	++
52	NS (EC)	+++
53	NS (TE, YS)	+
57	NS (EC)	+++
Semen (normal individuals) (n = 3)		−
Placenta (third trimester) (n = 1)		−
Testis parenchyma control individuals (n = 3)		−
Testis parenchyma next to tumor† (n = 10)		−

A total of 57 unilateral orchidectomy specimens suspected for the presence of a TGCT were given individual code numbers and classified histologically as described (16). Twenty-nine of these specimens, containing invasive TGCT, were analyzed for the expression of the 1.5-kb PDGF α-receptor transcript. In the case of NS both pure and mixed tumors were analyzed as indicated. Testis parenchyma of control individuals was obtained from patients who had died of reasons other than a TGCT.

*Quantification of expression levels: +++, detectable after 32 PCR cycles; ++, detectable after 42 PCR cycles; +, detectable only after 32 PCR cycles and an additional booster with 20 PCR cycles; +/−, detection not certain after booster PCR; −, not detectable after booster PCR.

†Testis parenchyma without the presence of CIS or invasive malignant component.

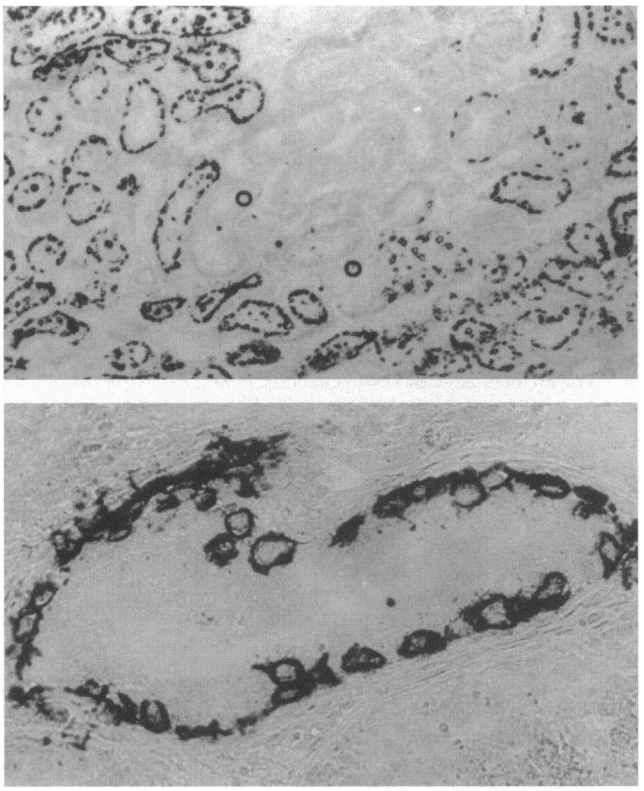

FIG. 3. Histological section of testis parenchyma containing CIS, stained for alkaline phosphatase. (*Upper*) Low-power magnification (×55) of testicular parenchyma containing seminiferous tubules without CIS in the central area, surrounded by a large number of tubules containing CIS. CIS cells are arranged as strings of beads. (*Lower*) High-power magnification (×180) of a single seminiferous tubule containing CIS.

number of PCR cycles required for detection of the transcript (see legend of Table 1). All negative controls were analyzed by the booster PCR protocol, as described. Although the present PCR analysis gives no quantitative information about the level of expression, it is striking that those NS which contain only EC cells and no differentiated components show detection at a lower number of cycles than those containing differentiated CH, TE, or YS components. This is in line with the observation that Tera-2 EC cells lose expression of the 1.5-kb transcript upon differentiation to mature cell types. The observed expression in the NS therefore most likely reflects the presence of undifferentiated EC stem cells in these mixed tumors.

Expression of 1.5-kb PDGF α-Receptor Transcript in CIS. Since CIS is the precursor of both SE and NS, the above observation prompted us to investigate if CIS cells also express the 1.5-kb transcript. CIS is generally present in seminiferous tubules adjacent to all histological types of TGCTs. From a number of the TGCTs, testis parenchyma was analyzed for the presence of CIS, as described in *Materials and Methods*. Fig. 3 shows a section containing CIS, stained for alkaline phosphatase. On the basis of the percentage of CIS-containing seminiferous tubules in the sections, the tissue was classified by a CIS index (see legend of Fig. 4). The data in Fig. 4 show that with a booster PCR the 1.5-kb PDGF α-receptor transcript could be detected in all CIS-positive samples, with a detection limit below 10% of CIS-positive tubules. Case 24, an atrophic testis without CIS, showed expression due to the presence of micro-invasive SE (not shown). Therefore, no false positive results were obtained. Although the PCR analysis cannot be considered to give quantitative results, a correlation was observed between the intensity of the PCR signal and the CIS index, again indicating that the CIS cells are most likely responsible for the expression of the 1.5-kb transcript. In addition, all controls and tissues from unrelated tumors, including Leydig cell tumor, were found to be negative for this marker. These data show that the 1.5-kb PDGF α-receptor transcript can be considered as an absolute marker for CIS cells of the testis and their derivatives.

DISCUSSION

In the present study we have shown that expression of the 1.5-kb PDGF α-receptor transcript is a highly selective marker for testicular germ cell tumors and their precursor, CIS. TGCTs are a significant cause of death for young adults, and the incidence of this type of tumor is still increasing (1, 2). As a consequence there is great need for a diagnostic method by which individuals at risk can be screened for early, preinvasive, stages of the disease (9). Existing methods rely on histological detection of CIS cells in testis parenchyma after surgical biopsy. However, CIS cells have also been detected in semen samples of tumor patients (21) and are also expected to be present in cell suspensions obtained by fine needle aspiration cytology of the testes of these patients (9). The present observation that CIS cells can be detected with high selectivity and PCR-based sensitivity opens the possibility of setting up diagnostic screening for CIS based on detection of the 1.5-kb PDGF α-receptor transcript in samples obtained by such non- or minimally invasive techniques.

Markers currently available for TGCTs can be subdivided into two categories; one is based on the embryonic origin of these cells, the other on specific initiation and progression

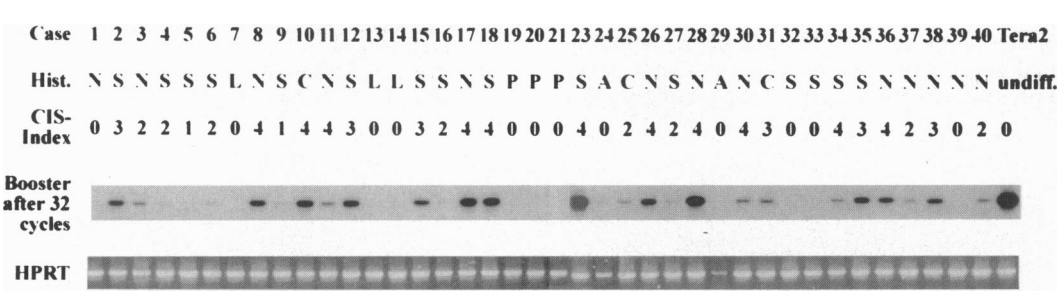

FIG. 4. Expression of the 1.5-kb PDGF α-receptor transcript in CIS. Testis parenchyma adjacent to TGCTs, but free of an invasive tumor component, was analyzed histochemically on sections for the fraction of seminiferous tubules containing CIS. This was quantified in a CIS index as follows: 0 (0% CIS), 1 (<10% CIS), 2 (10–40% CIS), 3 (40–75% CIS), and 4 (75–100% CIS). RNA was analyzed from parallel sections. The 1.5-kb transcript was detected by a booster PCR, with HPRT as RNA quality control. HPRT products are shown after staining of gel with ethidium bromide; 1.5-kb transcript PCR products, after hybridization of blots with an exon 16-specific probe (see legend of Fig. 1). The following abbreviations were used for histological characterization (Hist.): S, parenchyma adjacent to SE; N, parenchyma adjacent to NS; C, parenchyma adjacent to a combined tumor; P, normal testis; A, atrophic parenchyma; L, Leydig cell tumor. mRNA from Tera-2 EC cells was used as a positive control.

events during the tumorigenic process. The best-characterized tumor marker for TGCTs is the consistent overrepresentation of chromosome 12p sequences (22), while in addition monoclonal antibodies have been characterized which specifically recognize CIS cells in the testis (9). Using a combination of chromosomal and immunological markers for selecting cells by flow cytometry, attempts have been made to detect CIS cells in body fluids, but so far the diagnostic sensitivity of this method appears to be insufficient. Just as for alkaline phosphatase, the 1.5-kb PDGF α-receptor transcript is most likely already expressed in primordial germ cells. It is anticipated that expression of the 1.5-kb transcript is lost after the initial differentiation steps of the primordial germ cells to gonocytes in early life. If derangement of these primordial germ cells occurs, so that cellular differentiation is blocked, the derived CIS cells and TGCTs will still express the 1.5-kb transcript, until differentiation of NS stem cells to mature cell types takes place.

In the present study we have found evidence for four different PDGF α-receptor transcripts. The 6.4- and 3.0-kb transcripts are transcribed from a promoter upstream of exon 1 of the PDGF α-receptor gene (23), while the 5.0- and 1.5-kb transcripts appear to be transcribed from a promoter within intron 12 of the gene (13). Besides the 1.5-kb transcript, the tumors studied here generally also expressed the 5.0-kb transcript, which initiates from the same promoter but undergoes alternative splicing. Moreover, abundant expression of both the 6.4- and 3.0-kb transcripts was detected in all tissues, including the negative control samples presented in Table 1 and Fig. 4 (data not shown). Most likely these transcripts derive from stromal cells which have been removed with the tumor. In agreement with previous studies, we also found evidence for a 1.4-kb PDGF α-receptor transcript, which corresponds to the 1.5-kb transcript lacking exon 14 as a result of alternative splicing (13). Most likely exon 14-lacking variants exist for all four PDGF α-receptor transcripts described in this study.

The present data show that the 1.5-kb PDGF α-receptor transcript can be considered as a highly selective marker for TGCTs and CIS. Although we have tested only a limited number of control tissues so far, CIS cells appear to be the only cells expressing the 1.5-kb transcript in testis parenchyma. It may be possible to use the present RT-PCR analysis to set up a diagnostic assay for testing semen samples and cell suspensions obtained by aspiration cytology for the presence of CIS. Furthermore, the transcriptional regulation of the 1.5-kb transcript might be studied by analysis of the alternative promoter within intron 12 of the gene. Recent observations show that this second PDGF α-receptor promoter contains a functional binding site for the Oct family of transcription factors, while additional experiments indicate that Tera-2 cells express the Oct-4 gene only in the undifferentiated state (H.J.K., E. Piek, and E.J.J.v.Z., unpublished observation). Many early embryonic cells, including mouse primordial germ cells (24), express the Oct-4 gene, which may be a prerequisite for activity of the second PDGF α-receptor promoter. Interestingly, NS but not SE has been shown to express the FGF-4 gene, which is also known to be controlled by Oct-4 (25, 26).

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