

# Coamplification of *DAD-R*, *SOX5*, and *EKII* in Human Testicular Seminomas, with Specific Overexpression of *DAD-R*, Correlates with Reduced Levels of Apoptosis and Earlier Clinical Manifestation<sup>1</sup>

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## ABSTRACT

Seminomas and nonseminomas represent the invasive stages of testicular (TGCTs) of adolescents and adults. Although TGCTs are characterized by extra copies of the short arm of chromosome 12, the genetic basis for gain of 12p in the pathogenesis of this cancer is not yet understood. We have demonstrated that gain of 12p is related to invasive growth and that amplification of specific 12p sequences, *i.e.*, 12p11.2-p12.1, correlates with reduced apoptosis in the tumors. Here we show that three known genes map within the newly determined shortest region of overlap of amplification (SROA): *DAD-R*, *SOX5*, and *EKII*. Whereas *EKII* maps close to the telomeric region of the SROA, *DAD-R* is the first gene at the centromeric region within the 12p amplicon. Although all three genes are amplified to the same level within the SROA, expression of *DAD-R* is significantly up-regulated in seminomas with the restricted 12p amplification compared with seminomas without this amplicon. *DAD-R* is also highly expressed in nonseminomas of various histologies and derived cell lines, both lacking such amplification. This finding is of particular interest because seminomas with the restricted 12p amplification and nonseminomas are manifested clinically in the third decade of life and show a low degree of apoptosis. In contrast, seminomas lacking a restricted 12p amplification, showing significantly lower levels of *DAD-R* with pronounced apoptosis, manifest clinically in the fourth decade of life. A low level of *DAD-R* expression is also observed in normal testicular parenchyma and in parenchyma containing the precursor cells of this cancer, *i.e.*, carcinoma *in situ*. Therefore, elevated *DAD-R* expression in seminomas and nonseminomas correlates with invasive growth and a reduced level of apoptosis associated with an earlier clinical presentation. These data implicate *DAD-R* as a candidate gene responsible in part for the pathological effects resulting from gain of 12p sequences in TGCTs. In addition, our results also imply differences in expression regulation of *DAD-R* between seminomas and nonseminomas.

## INTRODUCTION

Invasive TGCTs,<sup>3</sup> the most frequent cancers in Caucasian males between the ages of 15 and 40 years (1), are clinically and histologically divided into seminomas and nonseminomas (2). Epidemiological data supported by morphological and immunohistochemical studies indicate that the initial event in the pathogenesis of this cancer

occurs during fetal development, leading to CIS (3–7). Several studies have provided insight into the progression of TGCTs. For example, genomic analyses have revealed that polyploidization is an early and crucial event, leading to invasive TGCTs with a total chromosomal content in the triploid range (8, 9). In addition to aneuploidy, the most consistent chromosomal anomaly found in invasive TGCTs is a relative gain of the short arm of chromosome 12, mediated by isochromosome 12p [i(12p)] formation in up to 80% of cases (see Refs. 10, 11 for review). The remaining 20% of i(12p)-negative TGCTs also contain additional copies of the short arm of chromosome 12 (12, 13). This finding indicates that an increased copy number of one or more genes located on 12p plays a role in the development of TGCTs. Although a gain of 12p sequences in invasive TGCTs was reported in 1982 (14), the underlying molecular basis for this condition has not yet been elucidated. We recently demonstrated that gain of 12p sequences is not detectable in CIS (15), indicating that overrepresentation of 12p is related to invasive growth of TGCTs. We further found that tumor cells derived from seminomas with a restricted 12p amplification, like nonseminomas, could be cultured for a few days *in vitro*, whereas seminomas lacking this amplification could not be grown in culture (16, 17). In fact, to date no cell lines of seminomas are available (18). Furthermore, patients with seminomas that carry a restricted 12p amplification are significantly younger at clinical presentation than other seminoma patients (17). In fact, they have the same age as patients with a nonseminoma.

These findings are consistent with the observation that seminomas with a restricted 12p amplification show reduced apoptosis compared with seminomas without such an amplification, whereas no differences in proliferation index are observed (17). We also showed that the presence of a restricted 12p amplification is related to invasive growth of the tumor cells *in vivo*. Overall, these data support a model whereby overexpression or ectopic expression of one or more genes on 12p permits survival of tumor cells in their invasive growth phase. Previous studies indicated that three known genes are mapped within the SROA on 12p in TGCTs: *JAW1*, *KRAS2*, and *SOX5* (16, 17). Although *JAW1* was not considered a gene of interest, because expression is most likely explained by the presence of infiltrating lymphocytes, no convincing data could be obtained supporting one of the other genes as candidate.

Here we extend the study on the restricted 12p amplification as found in TGCTs. We determined both the centromeric and telomeric breakpoints in more detail and investigated the copy numbers and expression levels of the genes mapped close to the breakpoints of the SROA. Interestingly, a gene is located at both the telomeric and centromeric side of the amplicon that is possibly involved in reduction of apoptotic cell death. Specifically, a novel intronless gene, designated *DAD-R*, recently assigned to the short arm of chromosome 12, band p11.2-p12.1 (19), maps the closest to the centromeric border of the amplicon and shows up-regulation in TGCTs with a low level of apoptosis. In fact, the newly determined breakpoint excludes *JAW1* and *KRAS2* from the SROA (16, 17). Although the precise function of

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<sup>3</sup> The abbreviations used are: TGCT, testicular germ cell tumor; CIS, carcinoma *in situ*; SROA, shortest region of overlap of amplification; DAD, defender against apoptotic cell death; FISH, fluorescence *in situ* hybridization; PAC, p1 artificial chromosome; BAC, bacterial artificial chromosome; STS, sequence-tagged site; RT-PCR, reverse transcription-PCR; HPRT, hypoxanthine phosphoribosyltransferase.

*DAD-R* is not yet known, a highly homologous gene, *DAD-1*, plays a role in apoptosis (20, 21) and is likely involved in *N*-glycosylation (22–24). Inactivation of *DAD-1* in mice results in induction of apoptosis, suggesting that the wild-type gene has a protective function. These mice show abnormal *N*-linked glycolipids (25). In addition, we demonstrate that *EKII* maps to the telomeric region of the restricted 12p amplification in TGCTs. This gene encodes ethanolamine kinase, which is the first committed step in phosphatidylethanolamine synthesis via the CDP-ethanolamine pathway (26). Interestingly, ethanolamine kinase-overexpressing cells are protected against apoptotic cell death (27). Our expression study shows that *DAD-R*, *SOX5*, and *EKII* are expressed at relatively low levels in testicular parenchyma samples and that the expression levels of *DAD-R* and *EKII* are independent of the presence of spermatogenesis or CIS. Although all three genes show higher expression levels in invasive TGCTs, *DAD-R* shows a specific and significant increased expression in seminomas with the restricted 12p amplification and nonseminomas without this amplification. The results also indicate that *DAD-R* is expressed mainly in TGCTs with a low intrinsic sensitivity to apoptosis and is therefore a candidate gene involved in the pathogenesis of TGCTs.

## MATERIALS AND METHODS

**Samples.** Freshly obtained tumor samples included in this study were collected in close collaboration with urologists and pathologists in the southwestern part of the Netherlands. All tumors were obtained before chemotherapy and/or irradiation. Directly after surgical removal, representative parts of the tumor and adjacent normal tissue (when available) were snap-frozen, and other tissue samples were fixed overnight in 10% buffered formalin and embedded in paraffin. The tumors were evaluated according to the WHO classification for testicular tumors (2). Tumors containing both a seminoma and a nonseminoma component were classified as combined tumors, according to the British classification (28), instead of nonseminomas according to the WHO classification system. Identification of CIS, seminoma, and embryonal carcinoma was aided by direct enzyme-histochemical detection of alkaline phosphatase activity on representative frozen tissue sections, as reported previously (29). Activated lymphocytes were prepared from *in vitro* phytohemagglutinin-stimulated peripheral blood lymphocytes cultures from three healthy males with (46,XY) karyotype, as described previously (30). Non-stimulated peripheral blood lymphocytes were harvested from a Ficoll gradient directly after collection. The cell lines used, NTera2, 2102Ep, and NCCIT, were cultured as reported previously (30, 31). RNA isolation and cDNA synthesis were performed as described below.

**FISH.** For each case analyzed, 4- $\mu$ m-thick frozen tissue sections were cut and air-dried at room temperature for 60 min on microscope slides treated with 3-aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, MO). In addition, two adjacent sections were used for histological examination. One was stained with H&E and the other for alkaline phosphatase activity. Slides for FISH were submerged in methanol-acetone (1:1, v/v) at  $-20^{\circ}$ C for 20 min, dehydrated in an increasing ethanol series (80, 90, and 100%; 2 min each), and air-dried. Subsequently, tissue sections were treated with 0.0005% pepsin (Sigma Chemical Co.) in 0.01 M HCl for 1 min at  $37^{\circ}$ C, washed five times for 1 min in water, and dehydrated. Hybridization was performed essentially as described for the methanol-acetic acid fixed nuclei (16). All probes used were labeled by nick translation. Probe 1A1, also known as 778I17 (mapping within the SROA, containing *SOX5*; see Fig. 1A) was used as a control probe in all experiments. Probe 1A1 was labeled with digoxigenin-11-dUTP (Roche, Mannheim, Germany) and visualized with FITC-conjugated sheep antidigoxigenin (Roche). A restricted 12p amplification was defined as the presence of  $\geq 15$  signals/interphase nucleus, as described previously (16, 17).

Newly identified TGCTs with a restricted 12p amplification (see "Results") underwent a more detailed analysis to determine the position of breakpoints. In these cases, the control probe was hybridized with several test probes (see Fig. 1A). Specifically, for the centromeric region probes containing *KRAS2* (cosmid clone), *JAW1* (PAC 524I18), and *DAD-R* (BAC 622I13), as well as a 20-kb centromeric end-clone derived from BAC 622I13 were used. For the telomeric region BAC 49G2, cosmid C34F8, BACs 449P1 and 268P4 were used. The

latter two clones contain the *EKII* gene. In the initial experiments, the control probe was labeled with digoxigenin-11-dUTP as described above, whereas test probes were labeled with biotin-16-dUTP and detected using avidin-CY3 (Jackson ImmunoResearch Laboratories). To verify the results, the labels on the probes were switched. The criteria used to determine the borders of the breakpoints were similar to those described previously (16, 17). Briefly, the test probe was scored as part of the amplicon when paired hybridization signals with the control probe were observed, and scored as outside the amplicon when the number of hybridization signals were found to be at least half compared with the control probe.

**Centromeric Clone Order Determination.** A contiguous sequence of 277 kb containing the complete sequences of the genes *JAW1* and *DAD-R* was assembled from the National Center for Biotechnology Information database.<sup>4</sup> This contig also contains the STSs D12S1617 and D12S1435. The sequence was generated based on the completely sequenced clones 713N11 (AC023510) and 662I13 (AC026310). The alignment was reconfirmed by medium- and long-range DNA fingerprinting and Southern blot hybridization of BACs 713N11 (AC023510) and 662I13 (AC026310). The probes used for the mapping were derived both from the ends and from internal restriction fragments of the aforementioned BAC clones. In addition, the order was confirmed using fiber-FISH on normal DNA, as described previously (32).

**Southern Blot Analysis of Breakpoints.** High-molecular weight DNA was isolated from a series of TGCTs with (six seminomas) and without (four seminomas, three embryonal carcinomas, two yolk sac tumors, and three teratomas) the restricted 12p amplification according to standard procedures (33). Genomic DNA was digested separately with *Eco*RI and *Bg*II and with a combination of both. After electrophoresis through a 0.6% agarose gel, the DNA was blotted onto Hybond N+ filters (Amersham). The filters were hybridized with a PCR generated cDNA probe (see section on semiquantitative RT-PCR below) labeled with [ $\alpha$ -<sup>32</sup>P]dATP. The expected sizes of the DNA fragments obtained from the digests were 15.1 kb for *Eco*RI, 4.1 kb for *Bg*II, and 3.2 kb for the double digest.

**Semiquantitative RT-PCR.** RNA was isolated from snap-frozen tissue samples with Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. For reference, the histological composition of the sample under investigation was checked by microscopic analysis of an adjacent section stained with H&E. The following samples were investigated: lymphocytes ( $n = 1$ ); normal testicular parenchyma ( $n = 5$ ); atrophic testicular parenchyma ( $n = 1$ ); testicular parenchyma with 20% CIS ( $n = 2$ ), 50% CIS ( $n = 2$ ), or 80% CIS ( $n = 2$ ); seminoma without the restricted 12p amplification ( $n = 4$ ); seminoma with the restricted 12p amplification ( $n = 11$ ); embryonal carcinoma ( $n = 3$ ); teratoma ( $n = 3$ ); and yolk sac tumor ( $n = 3$ ). All of these nonseminomas did not contain a restricted 12p amplification.

All RNA samples were pretreated with RNase-free DNase-I according to standard method (34). The RNA pellets were dissolved in diethyl pyrocarbonate-treated water. Complete removal of contaminating DNA is particularly important in this case because *DAD-R* lacks introns (19). The resulting RNA quality was checked by electrophoresis on a denaturing gel. To verify that the RNA samples were free of DNA, 0.5  $\mu$ g of total RNA was used as template in a PCR amplification with the primers DADR1a (5'-AGTGTCAAGGCAC-CCGATGG-3') and DADR2a (5'-GATGGTGCTAGCAAAGAGAAAG-3'), which generate a 293-bp fragment. These *DAD-R* primers differ from those used by Kuittinen *et al.* (19). For expression analysis of *SOX5* and *EKII*, the following primers were used: *SOX5F* (5'-TGCCTGGTGGATGGCAAA-AAGC-3') and *SOX5R* (5'-GTTAATGTGCTTGGCAC-3'), which generate a fragment of 477 bp (35), and *EKII1F2* (5'-TGGATCAAAGCATGTCTG-3') and *EKII1R3* (5'-TCATCTGCAAATCCTGTGGG-3'), which generate a fragment of 162 bp.

First-strand cDNA was synthesized from 1  $\mu$ g of oligo(dT) and random hexamer primed DNase-I-treated RNA in a total volume of 40  $\mu$ l according to standard procedures. The cDNA quality was checked by PCR with the primers HPRT 243 and HPRT 244, which amplify a specific 387-bp fragment from mRNA encoding the housekeeping gene *HPRT* (36).

For semiquantitative PCR, 0.5  $\mu$ l ( $\sim$ 12.5 ng of cDNA) of each reverse transcription reaction was amplified with 0.2  $\mu$ M each of the test primers, together with HPRT 243 and HPRT 244 in a volume of 50  $\mu$ l. In addition to

<sup>4</sup> <http://www.ncbi.nlm.nih.gov>.

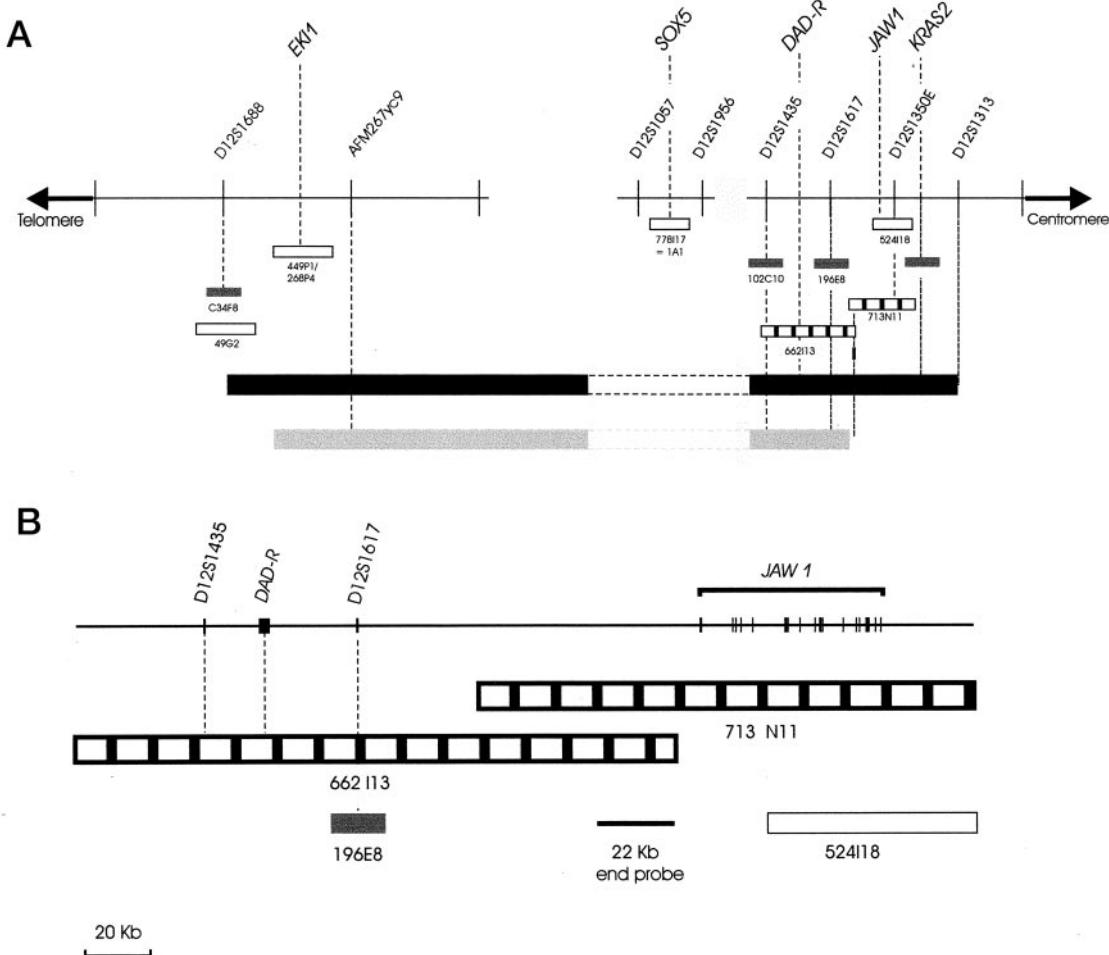


Fig. 1. A, schematic diagram of the centromeric and telomeric borders of the restricted 12p amplification found in invasive TGCTs in adolescents and adults. Genes, STSs, and probes used are shown. Cosmids are *C34F8*, *102C10*, *196E8*, and *KRAS2* (indicated by gray boxes), BACs and PACs are indicated by open boxes, and yeast artificial chromosomes by striped boxes. The thick black line indicates the region included in the restricted 12p amplification determined in our previous studies. The present study identified tumors in which the SROA is shorter, ~2.2 Mb in length, indicated by the thick gray line. The STSs, probes, and genes are not presented to scale. B, physical map of the centromeric breakpoint region of the SROA. The STSs, genes (including exons as black boxes), and probes are indicated to scale. Note the localization of the 22-kb end probe.

0.125 mM dNTPs, each PCR reaction was supplemented with 0.25  $\mu$ l of [ $\alpha$ -<sup>32</sup>P]dATP (10  $\mu$ Ci/ $\mu$ l). After an initial denaturation step of 2 min at 94°C, the cycling conditions used were as follows: 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s. Ten- $\mu$ l aliquots were taken during the amplification, typically after 17, 19, 21, and 23 cycles. Five  $\mu$ l of loading buffer were added to each aliquot, and 4  $\mu$ l were run on 4% native polyacrylamide gels. The gels were scanned on a Storm-820 Phosphor Imager (Molecular Dynamics), and the band intensities were quantified with the ImageQuant 5.0 Software.

We showed that the amplification products derived from *DAD-R* and not from *DAD-I* by digesting a 2- $\mu$ l aliquot from the last cycle of each sample with *Pst*I. This restriction endonuclease digests the amplification product of *DAD-R* twice. In addition, we used *Ava*I, which has no recognition site in *DAD-R*, in contrast to *DAD-I* (D15057; National Center for Biotechnology Information database). The expected digestion fragments of 186, 57, and 49 bp were obtained from the *Pst*I digestion, whereas the amplification products were not digested by *Ava*I. Expression levels of *DAD-R*, *SOX5*, and *EK11* were calculated as a ratio of *HPRT*. At least two consecutive data points that showed linear amplification of the products of both genes were chosen for the calculation. The final data were obtained from the results of at least two independent PCR reactions for each cDNA sample. The expression levels of *KRAS2*, *DAD-R*, and *HPRT* were analyzed by a triple RT-PCR on RNA derived from microdissected seminoma cells. The procedure was performed as described above, except that 0.20 mM rather than 0.125 mM was used for dGTP, dCTP, and dTTP, along with 0.15 mM dATP. The *KRAS2*-specific primers have been described (16). For analysis, samples were taken after 20, 21, 22, 23, 24, and 25 amplification cycles.

**Detection of Apoptosis Level.** The level of apoptosis was determined by electrophoresis of 1  $\mu$ g of high-molecular weight DNA isolated from snap-frozen histologically checked TGCTs with and without a restricted 12p amplification, as described previously (17). The presence of DNA laddering was visualized by ethidium bromide staining. Both seminomas with a restricted 12p amplification as well as seminomas and nonseminomas without a restricted 12p amplification were included in this analysis.

**Microdissection of Seminoma Cells.** Twenty- $\mu$ m-thick tissue sections from frozen samples were cut and stained for enzymatic alkaline phosphatase activity as reported (15) and air dried. The cells of interest were microdissected using the Arcturus Pixcell II system. Total RNA from the laser-captured microdissected samples was isolated by adding 500  $\mu$ l of Trizol reagent and incubating overnight at room temperature. RNA was DNase-I treated as described above, and the RNA pellet was dissolved in 10  $\mu$ l of Tris-EDTA. For the semiquantitative analysis, 2.5  $\mu$ l were used.

## RESULTS

**Presence of a Restricted 12p Amplification Correlates with a Younger Age of Patients at Clinical Presentation.** Our previous studies had suggested that patients with seminomas containing a restricted 12p amplification show clinical symptoms at a significantly younger age than patients without this anomaly (17). Therefore, we chose to analyze tissue samples from seminomas from patients diagnosed at an age younger than 30 years. These samples were screened

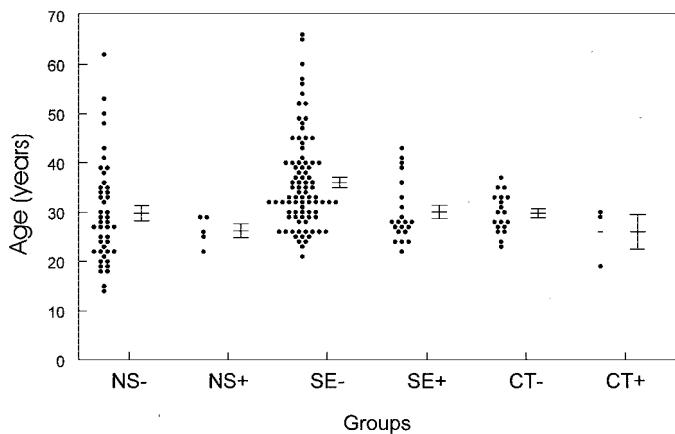


Fig. 2. Scatter plot of the ages at clinical presentation of patients with a nonseminoma without (NS-) and with (NS+) a restricted 12p amplification, seminoma without (SE-) and with (SE+) a restricted 12p amplification, or combined tumor without (CT-) and with (CT+) a restricted 12p amplification. Note the significant age difference between SE- and SE+, the latter being similar to NS- (– and +). Indicated are the ages at clinical presentation, mean and SE (bars). See text for statistics.

by FISH with a probe (1A1 = PAC 778I17, positive for *SOX5*) that maps approximately in the middle of the SROA (see Fig. 1A). Of 45 cases analyzed, 8 tumors showed an amplicon, amounting to a 16% incidence of seminomas with a restricted 12p amplification. This is a 2-fold increase compared with the general incidence in TGCTs, which is ~8% (16). To further investigate the relationship between the presence of a restricted 12p amplification and the age of the patients at clinical diagnosis, we tabulated the clinical data obtained from all of the patients we have analyzed for the presence of a restricted 12p amplification from our tumor bank according to age and histology of the tumors. The results are shown in Fig. 2 and indicate that patients with a 12p amplification-positive seminoma were significantly younger (mean age, 29.7 years) than those without the amplification (mean age, 36.0 years;  $P < 0.01$ ). In contrast, no age differences were observed in patients with a nonseminoma or a combined tumor (containing both a seminoma and a nonseminoma component) with and without a restricted 12p amplification. Patients with a seminoma containing a restricted 12p amplification were of the same age at clinical diagnosis as patients with a nonseminoma.

**Refinement of SROA and Localization of DAD-R.** We have previously shown that the centromeric and telomeric breakpoints cluster to relatively small regions (17). To define more precisely the

breakpoints of the amplicon, we have analyzed a total of 21 seminomas and 5 nonseminomas with a restricted 12p amplification identified previously (Refs. 16, 17 and in this study). The copy numbers of the amplicon, as identified by FISH with the 1A1 probe, varied between 15 and 50 per nucleus on tissue sections. The majority of tumors showed 20–35 copies/interphase nucleus on a tissue section. The centromeric border of the amplicon was mapped with a series of probes moving inward from *KRAS2* toward the center of the amplicon. A total of five probes were used (Fig. 1 and “Materials and Methods”). The results indicate that 9 of 21 seminomas (43%) and 1 of 5 nonseminomas (20%) with a restricted 12p amplification had at least two times more copy numbers of the sequences that are part of BAC 662I13 and the cosmid probe for STS D12S1617 compared with the control probe (see Fig. 3). The probability that this occurs by chance in the group of seminomas is  $1.29 \times 10^{-20}$  (see Ref. 17 for calculation). Analysis of the public databases showed that the region around the centromeric breakpoint of the amplicon is gene poor, but more interestingly, they indicate that *DAD-R*, a newly identified gene, mapped only ~20–25 kb from the breakpoint. Southern blot analysis (using a cDNA PCR amplification product as probe) of seminomas with and without a restricted 12p amplification and a series of nonseminomas without the amplification showed the absence of any aberrant genomic bands, indicating the absence of rearrangements within a 16–20-kb region near the *DAD-R* coding sequence (data not shown).

At the telomeric side of the amplicon a similar approach was performed. Here, four different probes in combination with the control probe (1A1) were used (see Fig. 1A). Whereas four seminomas showed at least half the number of signals when C34F8 was used as probe compared with the control probe, this was the case for only one when 49G2 was used as probe. These results were confirmed with the other two probes (449P1 and 268P4) as well as with the same probes, but labeled conversely. In total, 4 of the 21 seminomas (19%) showed the telomeric breakpoint between C34F8 and 449P1/268P4. The probability for this phenomenon by chance is  $7.2 \times 10^{-6}$ . These results indicate that the breakpoints at the telomeric side of the amplicon are less, although still highly significantly clustered than those at the centromeric side (see above). In fact, one of the tumors had the SROA as the actual amplicon. The first known gene present within the SROA at the telomeric side was found to be *EK11*. Therefore, in the seminoma with the smallest amplified fragment, *EK11* and *DAD-R* are located at the borders of the amplified region.

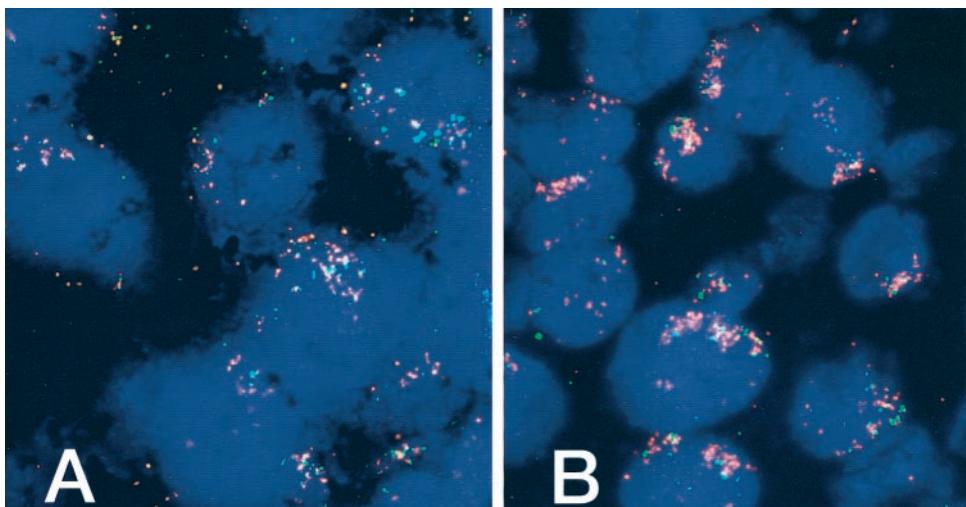


Fig. 3. A, representative example of FISH analysis of a frozen tissue section of a seminoma with a restricted 12p amplification, using the control probe (1A1, in red) and a probe positive for *KRAS2* (in green), indicating a lower copy number of the latter compared with the former. B, representative example of FISH analysis of the same tumor shown in A, using the control probe (1A1, in red) and the centromeric end probe BAC 662I13 (in green; see also Fig. 1), indicating a lower number of hybridization signals of the latter compared with the former.

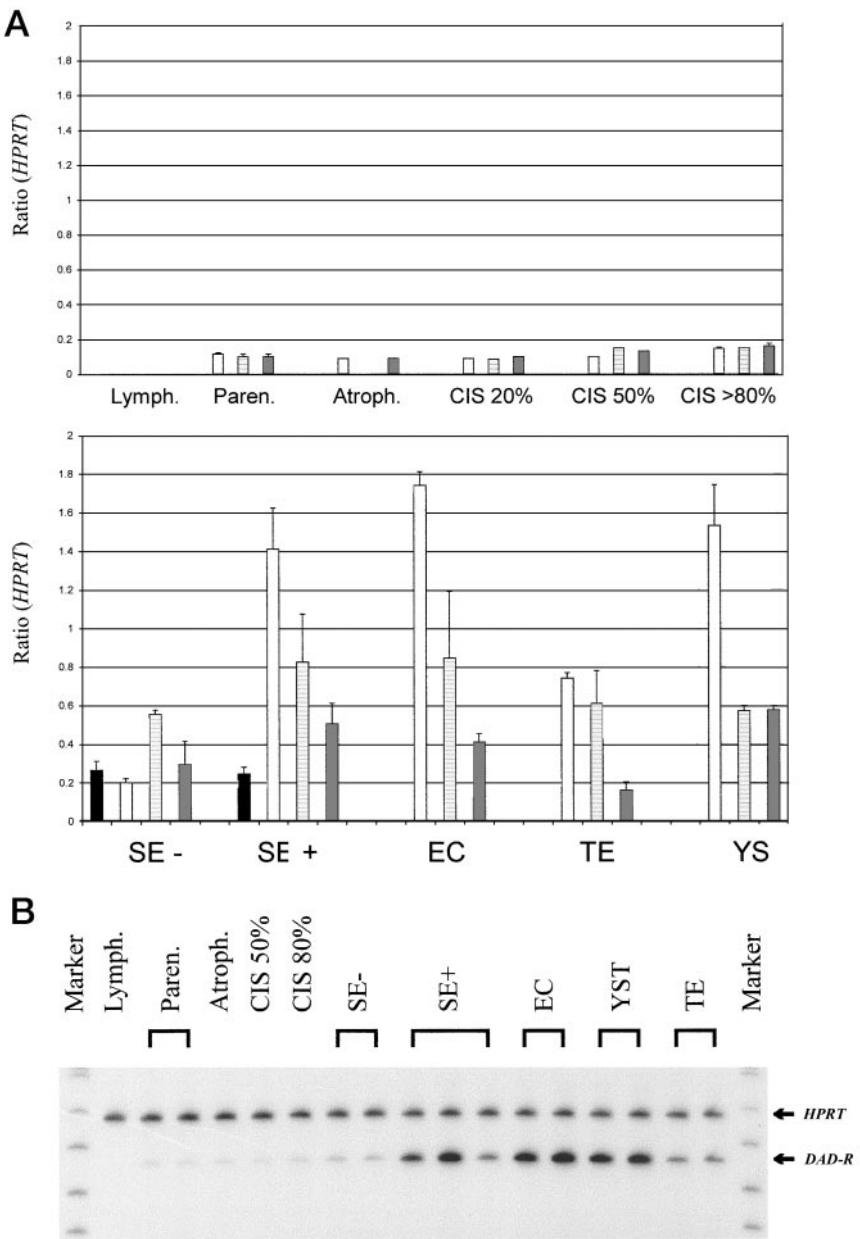


Fig. 4. A, summary of the expression analysis of *DAD-R* (open columns), *SOX5* (hatched columns), *EKII* (dark gray columns), and *KRAS2* (black columns) by semiquantitative RT-PCR. The samples studied are activated lymphocytes (Lymph.), normal testicular parenchyma (Paren.), atrophic testicular parenchyma (Atroph.), testicular parenchyma with various amounts of CIS (CIS) containing seminiferous tubules (20, 50, and >80%), seminomas without a restricted 12p amplification (SE-), seminoma with a restricted 12p amplification (SE+), and nonseminomas of various histologies (EC, embryonal carcinoma; TE, teratoma; YS, yolk sac tumor). Indicated are the ratios of expression of the target gene *versus* *HPRT*, mean and SD (bars). B, representative examples of *DAD-R* expression of the various groups tested (see legend for A for abbreviations used, except YST, yolk sac tumor).

**Expression Analysis of *DAD-R*, *SOX5*, and *EKII*.** Because of the significant clustering of breakpoints at both the centromeric and telomeric borders of the restricted 12p amplification, the expression levels of the two genes mapped close to the borders, as well as *SOX5*, were analyzed in TGCTs with various histological compositions and gene copy numbers and a series of control samples by semiquantitative RT-PCR. The results of expression analysis are summarized in Fig. 4A, and representative examples for *DAD-R* are shown in Fig. 4B. The control samples showed a low level of expression of *DAD-R*, *SOX5*, and *EKII*. Transcripts were detected in activated lymphocytes only after 28 PCR amplification cycles. Because the detection level was below the linear range used to quantify the other samples, we concluded that the contribution of RNA from these genes to the RNA from lymphocytes in the samples under investigation is minimal. A relatively low but quantifiable level of expression was detected in testicular parenchyma with spermatogenesis. The expression levels of *DAD-R* and *EKII* were similar to those detected in an atrophic testicular parenchyma sample. In contrast, no expression of *SOX5* was observed in atrophic testis, which is expected because this gene is

specifically expressed during spermatogenesis (35). No significant differences in the expression levels of these genes were found in testicular parenchyma samples with various amounts of CIS. In contrast, all invasive TGCTs showed significantly higher expression levels for all three genes within the SROA compared with the different testicular parenchyma samples ( $P < 0.001$ ). However, only *DAD-R* showed a significant difference in expression level between the different histological groups of TGCTs. Seminomas without a restricted 12p amplification showed the lowest expression level, whereas a significantly higher level of expression was found in seminomas with a restricted 12p amplification ( $P < 0.0077$ ). Only one seminoma did not show enhanced expression (ratio, 0.16). Interestingly, this case was found to be more similar to seminomas without a restricted 12p amplification, as determined by cDNA array analysis.<sup>5</sup> One case showing intermediate expression (ratio, 0.37) was found to contain a significant component of testicular parenchyma in

<sup>5</sup> Unpublished results.

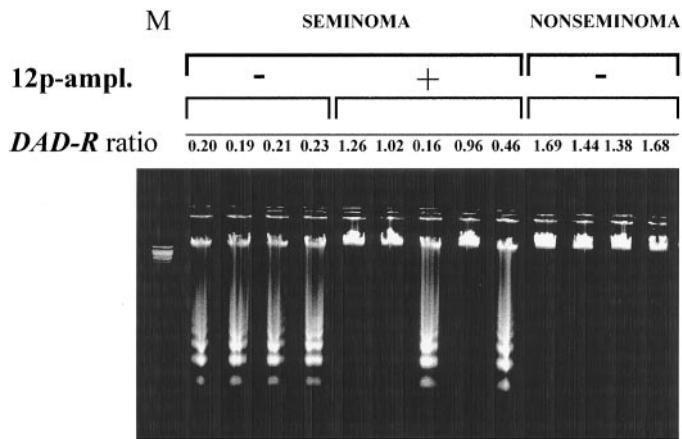


Fig. 5. Representative examples of the level of apoptosis as demonstrated by the presence of DNA laddering after gel electrophoresis of 1  $\mu$ g of DNA and ethidium bromide staining in seminomas without a restricted 12p amplification (left-hand lanes), with a restricted 12p amplification (middle lanes), and nonseminomas (right-hand lanes). The expression ratios for *DAD-R* (ratio versus *HPRT*) are indicated. *M*, marker.

the sample used for analysis. In one case, both the seminoma component with the restricted 12p amplification and its adjacent parenchyma (with >80% CIS-containing seminiferous tubules) were investigated separately. In this specimen, high *DAD-R* expression was detected only in the invasive component. These data support the hypothesis that up-regulation of *DAD-R* expression is related to invasive growth in TGCTs. Furthermore, none of the nonseminomas tested (three embryonal carcinomas, two teratomas, and three yolk sac tumors) contained a restricted 12p amplification, but all showed higher *DAD-R* expression compared with testicular parenchyma and seminomas without a restricted 12p amplification. In fact, the level of expression was similar to that found in seminomas with the restricted 12p amplification. The lower *DAD-R* expression level in teratomas was likely attributable to the low number of tumor cells in the samples as judged from histology.

***DAD-R* and Apoptosis.** We demonstrated previously that seminomas with a restricted 12p amplification showed a reduced level of apoptosis compared with seminomas without a restricted 12p amplification (17). Here we confirm this finding by investigating a total of 15 seminomas [9 reported previously by this group (17)] with a restricted 12p amplification, and we correlate this with *DAD-R* expression. Twelve of these tumors (80%) showed a low level of apoptosis, detected by the presence of DNA laddering upon gel electrophoresis. Eight of these were studied for the level of *DAD-R* expression and showed a high expression, with the exception of one (ratio, 0.37). This latter case contained a significant amount of testicular parenchyma (see above). The two cases with a profound level of apoptosis studied for *DAD-R* showed a low level of *DAD-R* expression (ratios, 0.16 and 0.46). In other words, all seminomas with a restricted 12p amplification that had a *DAD-R* expression ratio >0.76 ( $n = 7$ ) also showed a low level of apoptosis. This correlation between level of *DAD-R* expression and apoptosis was further supported by the profound level of apoptosis in seminomas without a restricted 12p amplification and with a low level of *DAD-R* expression ( $n = 4$ ; average ratio, 0.20; SD, 0.02) and the low level of apoptosis in case of high *DAD-R* expression in nonseminomas ( $n = 9$ ), of which representative examples are shown in Fig. 5, as well as the findings in the nonseminomatous cell lines (see below).

**Expression Levels of *KRAS2* versus *DAD-R*.** To study the relationship between expression levels of *DAD-R* and *KRAS2*, which was included in the amplified fragment in the majority of the TGCTs (see above), triple semiquantitative RT-PCR including *KRAS2*, *DAD-R*,

and *HPRT* was performed. RNA was isolated from seminoma cells microdissected from frozen tissue sections and stained enzymatically for alkaline phosphatase to identify tumor cells (see Fig. 6A). This is because activated lymphocytes also show expression of *KRAS2* (data not shown). Both seminomas without ( $n = 3$ ) and with ( $n = 5$ ) a restricted 12p amplification were investigated, as demonstrated by FISH with the control probe on an adjacent section (not shown). All of the seminomas with a restricted 12p amplification showed increased *DAD-R* expression (see above), which confirms the above-mentioned results, which are based on nonmicrodissected material. The results of the expression analyses are summarized in Fig. 6B, and representative examples are shown in Fig. 6C. Note that no differences in *KRAS2* expression were observed between seminomas with and without a restricted 12p amplification (mean ratio of *DAD-R* to *HPRT*, 0.23 and 0.28, respectively). In contrast, the *DAD-R* expression level was enhanced in the presence of an amplification. These data demonstrate that the expression level of *KRAS2* is not influenced by the presence of a restricted 12p amplification, like *SOX5* and *EK11*, indicating that alterations in expression levels of these genes cannot account for the biological differences observed between seminomas with and without a restricted 12p amplification.

**Expression Analysis of *DAD-R*, *SOX5*, and *EK11* in TGCT-derived Cell Lines.** To further investigate the expression of these genes in nonseminomas, we studied a series of representative cell lines derived from nonseminomas. The copy numbers of the genes to be investigated were determined by FISH and the expression levels by semiquantitative RT-PCR. The results of the copy number analyses are summarized in Table 1 and are in accordance to our previous findings (30). The NTera2 cell line has two normal chromosomes 12 and two isochromosomes 12p. There are four normal chromosomes 12 and one isochromosome 12p in 2102Ep. The cell line NCCIT has three to four normal chromosomes 12 and one derivative. The expression levels of the three genes mapped within the SROA are also shown in Table 1 and indicate that expression of *DAD-R* is the highest, being at a level similar to that of *HPRT*. No changes in expression levels were observed by induction of differentiation by retinoic acid. This supports the finding that the lower expression of these genes detected in the clinical samples composed of teratomas (see above) is attributable to the smaller amount of tumor cells present in the samples analyzed.

## DISCUSSION

Identification of potentially relevant genes that map to the short arm of chromosome 12 is a major goal in the study of the pathogenesis of TGCTs. Because of the consistent observation of additional copies of 12p in invasive TGCTs, it is likely that extra copies of genes on 12p are a requirement for the tumor to become clinically overt. We have proposed that in the progression from CIS to invasive tumor, genes on 12p prevent induction of apoptosis of tumor cells when they leave the seminiferous tubules. This hypothesis was derived from analysis of TGCTs with a restricted 12p amplification (16, 17). This region of amplification is tandemly organized on one chromosome, and it is not distributed throughout the genome (17, 37), which was shown both by spectral karyotyping as well as by FISH using region-specific probes. In addition, this has recently been supported by our finding that CIS lacks a gain of 12p sequences (15). The study presented here is in agreement with this model and identifies a putative candidate gene. In a series of TGCTs with a restricted 12p amplification, we found that the breakpoints of the amplicons clustered significantly to relatively narrow regions at both the telomeric and centromeric ends (17). This observation, confirmed in this study, suggests the presence of relevant genes in those particular areas on which we focused in this study. Our

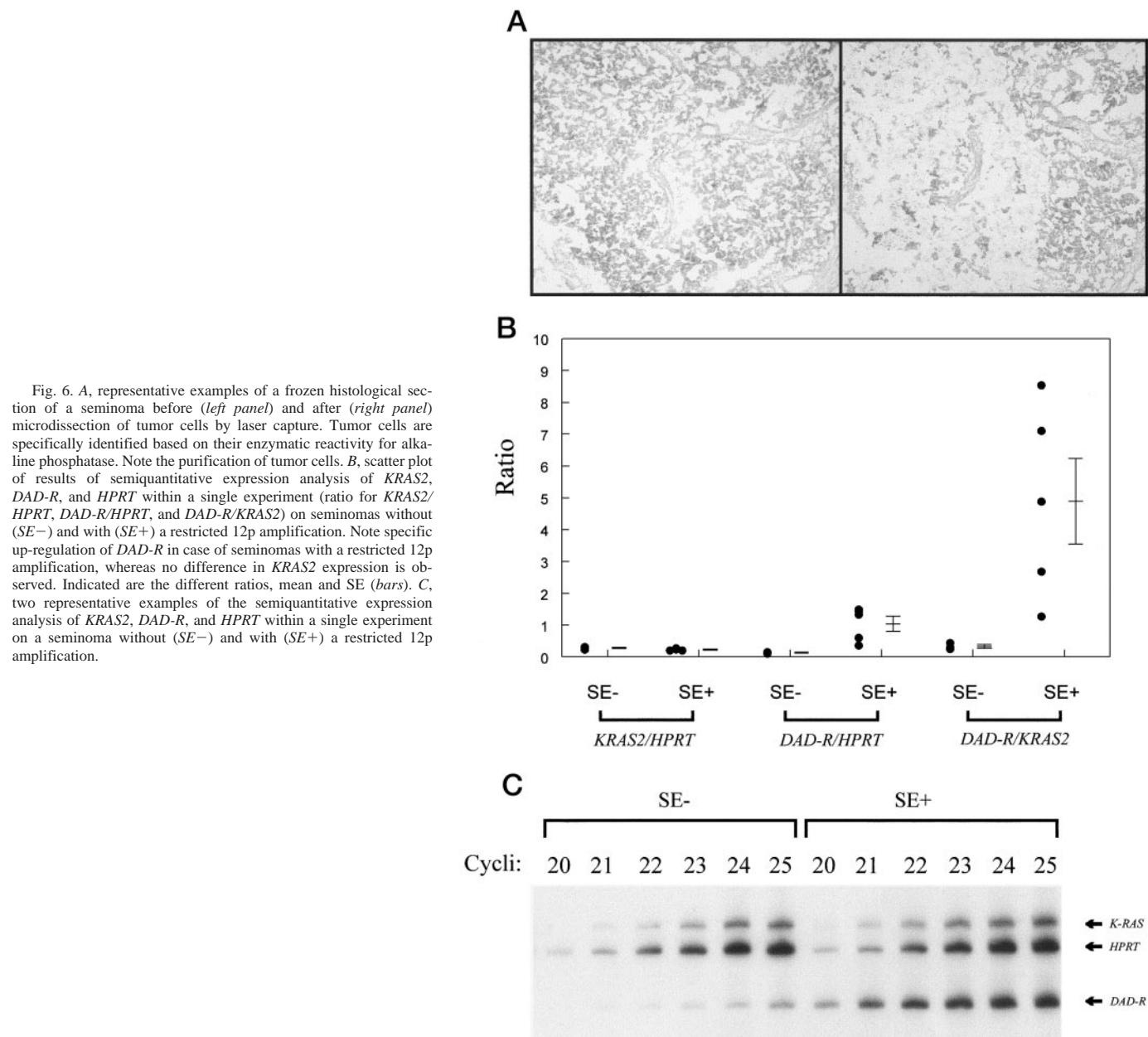


Fig. 6. A, representative examples of a frozen histological section of a seminoma before (left panel) and after (right panel) microdissection of tumor cells by laser capture. Tumor cells are specifically identified based on their enzymatic reactivity for alkaline phosphatase. Note the purification of tumor cells. B, scatter plot of results of semiquantitative expression analysis of *KRAS2*, *DAD-R*, and *HPRT* within a single experiment (ratio for *KRAS2*/*HPRT*, *DAD-R*/*HPRT*, and *DAD-R*/*KRAS2*) on seminomas without (*SE-*) and with (*SE+*) a restricted 12p amplification. Note specific up-regulation of *DAD-R* in case of seminomas with a restricted 12p amplification, whereas no difference in *KRAS2* expression is observed. Indicated are the different ratios, mean and SE (bars). C, two representative examples of the semiquantitative expression analysis of *KRAS2*, *DAD-R*, and *HPRT* within a single experiment on a seminoma without (*SE-*) and with (*SE+*) a restricted 12p amplification.

previous investigations revealed that three known genes map to the SROA: (from centromeric to telomeric) *KRAS2*, *JAW1*, and *SOX5* (16, 17), of which *KRAS2* has been suggested to be the most likely candidate (38). The mapping data presented in this report have excluded both *KRAS2* and *JAW1* from the SROA, thereby making them less likely candidates. These data are in accordance to our *KRAS2* expression analysis, which shows that the presence of a restricted 12p amplification does not affect the expression level of this gene, indicating that differences in *KRAS2* expression cannot explain the biological differences between seminomas with and without a restricted 12p amplification. In addition, the putative role of *KRAS2* in malignant transformation has been questioned because of the discrepancy in expression levels needed in *in vitro* and *in vivo* systems (39).

Two additional candidates mapping close to the borders of the SROA have been identified in this study. We demonstrate that *DAD-R* maps close to the centromeric border of the SROA. This gene is highly homologous to *DAD-1*, which suppresses induction of apoptosis (21, 22). In addition, *EK11* maps to the telomeric border of the

SROA, and overexpression of this gene has also been found to suppress apoptotic cell death (40). Because of their localization and possible functions, we investigated the copy numbers of these genes within the restricted 12p amplification and determined their level of

Table 1 Results of the semiquantitative expression analysis (ratio to *HPRT*) of *EK11*, *SOX5*, and *DAD-R* in a series of TGCT cell lines, before and after retinoic acid differentiation induction

Histology	Gene <sup>a</sup>		
	<i>EK11</i>	<i>SOX5</i>	<i>DAD-R</i>
NTera2			
-RA <sup>b</sup>	0.42 (7)	0.75 (7)	1.15 (7)
+RA	0.40 (7)	0.80 (7)	1.10 (7)
2102EP			
-RA	0.38 (6)	0.65 (6)	1.10 (6)
+ RA	0.39 (6)	0.70 (6)	1.05 (6)
NCCIT	0.41 (4-6)	0.60 (4)	1.15 (4)

<sup>a</sup> Gene copy number in parentheses.

<sup>b</sup> RA, retinoic acid.

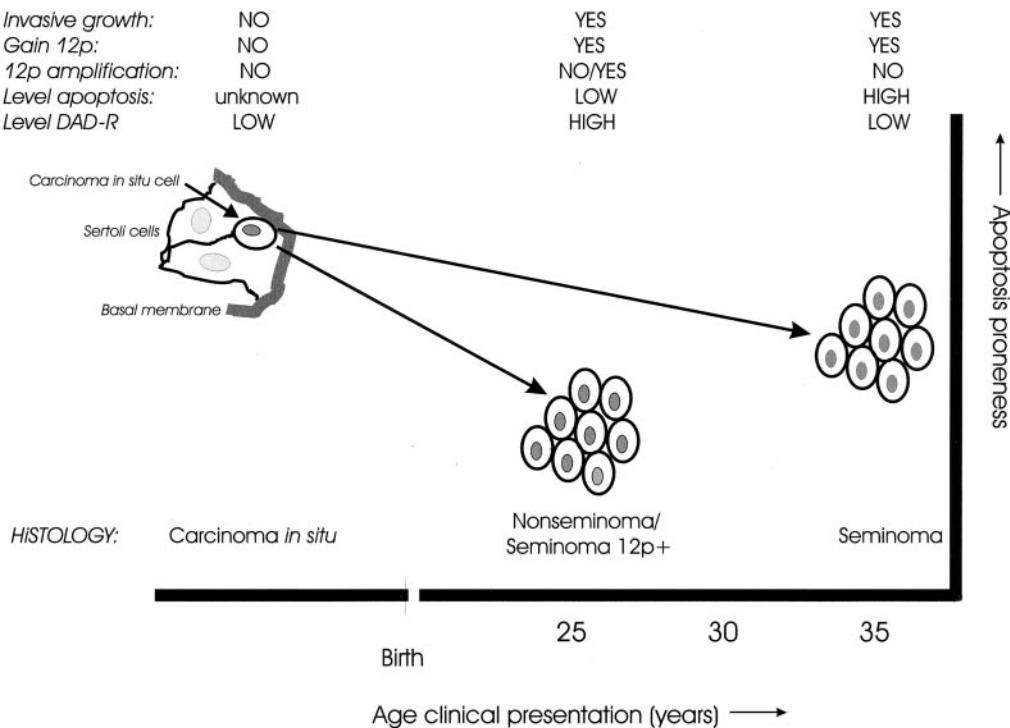


Fig. 7. Schematic representation of the model describing the role of gain and amplification of 12p in the development of TGCTs in adolescents and adults. The initiating event occurs during prenatal development. This event affects an embryonic germ cell (likely a primordial germ cell), leading to CIS, which is localized to the inner side of the basal membrane of seminiferous tubules under the tight junctions between Sertoli cells. CIS lacks gain and restricted amplification of 12p sequences. In contrast, all invasive TGCTs, both seminomas and nonseminomas, have extra copies of 12p, either by gain or a restricted amplification, which allow invasive growth. Whereas seminomas normally manifest themselves in the fourth decade of life, nonseminomas occur in the third decade of life. The higher level of apoptosis of seminomas compared with nonseminomas might explain this age difference. The level of apoptosis inversely correlates with the expression levels of *DAD-R*, being low in seminomas and high in nonseminomas. Seminomas with a restricted 12p amplification present clinically within the third decade of life and, like nonseminomas, show a low level of apoptosis and high expression level of *DAD-R*. Our data implicate *DAD-R* as the most likely candidate gene to mediate the role of amplification of 12p in invasive TGCTs. Increased expression of *DAD-R* results in a more profound inhibition of induction of apoptosis, allowing better survival of the tumor cells outside the specific microenvironment of the seminiferous tubules. The expression level of *DAD-R* in nonseminomas is less dependent on the copy number of the gene, which may explain the higher incidence of restricted 12p amplification in seminomas compared with nonseminomas.

expression in the various histological variants of TGCTs and control samples. No enhanced expression of these genes occurs in CIS-containing parenchyma compared with normal and atrophic testicular parenchyma. In contrast, all invasive TGCTs show significantly higher levels of *DAD-R* mRNA and, to a lesser extent, of *EKII* and *SOX5*. This indicates that the levels of expression of these genes are related to invasive growth of this cancer, in accordance to our previous finding that gain of 12p is restricted to invasive TGCTs (15). Although overexpression of *EKII* might prevent apoptotic cell death (27), it seems not to be the most important gene within the SROA, because no significant increase in expression related to copy numbers of the gene was observed. Most striking is the finding that seminomas with a restricted 12p amplification show specifically and significantly higher expression levels of *DAD-R* (not of *SOX5*, *EKII*, or *KRAS2*) compared with seminomas without this amplification. This is despite the similar copy numbers of the three genes within the SROA. The difference in expression level of *DAD-R* between seminomas without and with a restricted 12p amplification, ~0.2 and 1.4 times the level of *HPRT* (~7-fold increase), is expected based on the copy numbers per nucleus found. Seminomas without a restricted 12p amplification contain in general 5–6 copy numbers of 12p per cell (10), whereas seminomas with a restricted 12p amplification show 15–50 copy numbers/nucleus on 4-μm-thick tissue sections (see "Materials and Methods").

Interestingly, a expression level of *DAD-R* similar to that found in the seminomas with the restricted 12p amplification was detected in nonseminomas and derived cell lines, which lack a restricted 12p

amplification. On average, nonseminomas, and the derived cell lines have six to seven copies of 12p per nucleus (10, 30).

The specific clustering of breakpoints at the centromeric side of the amplicon, in contrast to a more heterogeneous, but still significant, pattern at the telomeric side, supports the importance of *DAD-R* in the pathogenesis of TGCTs. Noteworthy in this context is the finding that patients with a seminoma containing a restricted 12p amplification present at a age similar to that of patients with a nonseminoma (Ref. 17 and this study). Moreover, seminomas with a restricted 12p amplification as well as nonseminomas can be grown *in vitro* for an extended period of time, and both are more resistant to apoptosis (Refs. 17, 41 and this study). Neither of these conditions were found for seminomas without a restricted 12p amplification, which present at an older age. However, no differences in stage of the disease and treatment sensitivity of the cancer that depend on the presence of a restricted 12p amplification have been found (17). These results indicate that the level of *DAD-R* expression is related to the likelihood of apoptosis, thereby influencing the age of clinical manifestation of the tumor and, probably by the same mechanism, the ability of the tumor cells to survive *in vitro*. This model is schematically shown in Fig. 7. Our data also indicate the existence of a different regulatory mechanism that controls expression of *DAD-R* in seminomas than in nonseminomas of various histologies, the latter being independent of copy number. *DAD-R* expression does not seem to be influenced by differentiation status, as confirmed with the cell lines tested before and after differentiation induction by retinoic acid.

To date, this is the first report that links the level of expression of *DAD-R* with a biological phenomenon. However, no direct proof is as yet available that this gene encodes a functional protein. To better elucidate the function of this gene, we are assessing the function of *DAD-R* in model systems and determining the subcellular localization of its protein product immunologically.<sup>6</sup> Furthermore, we have preliminary data that show that the coding sequences of *DAD-R* are evolutionary conserved.<sup>7</sup>

In conclusion, we have demonstrated that the restricted 12p amplification as present in a selected series of TGCTs contains at least three genes, *DAD-R*, *SOX5*, and *EKII*. *DAD-1* maps the closest to the centromeric border of the SROA, and *EKII* to the telomeric border. The highest clustering of breakpoints was found at the centromeric breakpoint, close to *DAD-R*. Despite similar copy numbers within the restricted 12p amplification, the expression of *DAD-R* is specifically up-regulated, being associated with a reduced level of apoptosis. It remains to be elucidated whether overexpression of *DAD-R* might influence the pattern of *N*-glycosylation, as found for *DAD-1* (25). We demonstrated that seminomas indeed have a pattern of glycoproteins different from that of nonseminomas (42), which might be related to this phenomenon. These findings, together with our earlier results, point to *DAD-R* as a likely candidate gene able to explain the advantage of accumulating extra copies of 12p in the establishment of invasive TGCTs.

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<sup>6</sup> G. Zafarana, manuscript in preparation.

<sup>7</sup> Unpublished observation.

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