Identification of genes differentially expressed in testes containing carcinoma in situ


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Virtually all testicular germ cell tumours originate from a common precursor, the carcinoma in situ (CIS) cell. The precise nature of the molecular mechanisms leading to CIS remains largely unknown. We performed the first systematic analysis of gene expression in testis with CIS compared to normal testis by the differential display (DDRT–PCR) method, with subsequent analysis by RT–PCR and in situ hybridization (ISH). In tissue containing CIS we identified overexpression of 28 mRNA, some previously reported in CIS and a number of genes not previously described in germ cell neoplasia, including the novel expressed sequence tag (EST) OIC1 (Overexpressed In CIS). The genes could be grouped functionally into genes involved in cell growth, proliferation, differentiation, immunological response, and genes with unknown biological function. Examples of overexpressed genes are SFRP1 that is involved in Wnt signalling and IGFBP6, which is of importance for fetal growth and inhibits cell growth through insulin-like growth factor-II. ISH analysis showed that both mRNA were localized to CIS cells. The results of our search for differentially expressed genes in CIS demonstrated a number of genes linked to testicular development (e.g. DCN, IGFBP6, SFRP1, SALL1), supporting our hypothesis that the origin of CIS is probably associated with disturbances of the fetal development of the testis.

Key words: carcinoma in situ/differential display/IGFBP-6/SFRP1/testicular cancer

Introduction

Testicular germ cell tumours (TGCT) are the most common malignancies among men aged 17–45 years, and the incidence has been steadily increasing (Adami et al., 1994). TGCT of young adults are classified into two main histological subtypes, classical seminoma and non-seminoma. These tumours originate from a common precursor, the carcinoma in situ (CIS) cell (Skakkebaek, 1972), which is believed to arise by transformation of a gonocyte (Skakkebaek et al., 1987). Epidemiological evidence and comparative studies of cell surface proteins indicate that CIS is an inborn lesion, probably arising in early fetal life, which progresses to an overt TGCT after puberty. The precise nature of the molecular mechanisms leading to CIS remains largely unknown. Morphologically, CIS cells are gonocyte-like, intratubular germ cells that share several features with seminoma and to a lesser extent with embryonal carcinoma.

Several genes have been shown to be highly expressed in CIS, e.g. KIT (c-kit), CCND2, POU5F1, ALPP, mainly by studies using the candidate gene approach and immunohistochemistry (reviewed recently in Rajpert-De Meyts et al., 2003). No systematic studies of gene expression in CIS have been performed so far, partly due to technical difficulties in working with CIS, as these cells occur in low numbers scattered within normal testicular tissue. A few previous studies analysed gene expression profiles in overt testicular tumours, with focus on genes on chromosomes 17 and 12, because of frequent rearrangements of these chromosomes in human cancers (Rothe et al., 2000; Skotheim et al., 2002, 2003; Rodriguez et al., 2003).

To shed more light on CIS cell biology and tumour progression, we decided to analyse gene expression in CIS cells and the most common overt TGCT. Our additional objective in this study was to identify possible new markers for early detection of CIS, therefore the focus was on genes overexpressed in CIS in comparison to normal testicular parenchyma. We used the differential display (DDRT–PCR) method, in order to be able to detect not only known genes but also new expressed sequence tag (EST) in a truly unselected approach, followed by further analysis by reverse transcriptase PCR (RT–PCR) and determination of the cell type-specific expression of selected genes by in situ hybridization (ISH).

Materials and methods

Tissue samples

The testicular tissue samples (Table I) were obtained directly after orchidectomy and macroscopic pathological evaluation. Each testicular sample was divided into several tissue fragments: two or three fragments were snap-frozen at −80°C for nucleic acid extraction, and several adjacent fragments were fixed overnight at 4°C in Stieve’s fluid, and subsequently embedded in paraffin and stained with haematoxylin and eosin (HE) or with antibodies against placental alkaline phosphatase (PLAP or ALPP) for histological evaluation (Givercman et al., 1991). The use of the testicular tissue samples was approved by the Regional Committee for Medical Research Ethics in Denmark.

DDRT–PCR screening

Total RNA was prepared using the RNeasy kit from Qiagen as described by the manufacturer (Qiagen, Germany). RNA samples were double DNAse-digested, first ‘on-column’ and, after the RNA had been purified, in solution. cDNA was prepared using one-base-anchored AAGCT₁V (V = A, C or G) downstream

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Table I. Clinical and pathological data on testicular tissue samples

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Histology</th>
<th>In the vicinity of:</th>
<th>Usage</th>
<th>Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIS1</td>
<td>CIS 100%, some microinfiltration, atrophy</td>
<td>Embryonal carcinoma</td>
<td>DD</td>
<td>27</td>
</tr>
<tr>
<td>CIS2</td>
<td>CIS 100%, some microinfiltration, atrophy</td>
<td>No tumour, intraabdominal tests</td>
<td>DD, RT-PCR</td>
<td>35</td>
</tr>
<tr>
<td>CIS3</td>
<td>CIS 95%, 5% normal tubules</td>
<td>Emorycinoma</td>
<td>RT-PCR</td>
<td>26</td>
</tr>
<tr>
<td>CIS4</td>
<td>CIS 100%, microlihiasis, undifferentiated tubules</td>
<td>Classical seminoma</td>
<td>RT-PCR</td>
<td>27</td>
</tr>
<tr>
<td>CIS5</td>
<td>CIS 95%, 5% normal tubules</td>
<td>Classical seminoma</td>
<td>RT-PCR</td>
<td>33</td>
</tr>
<tr>
<td>SEM1</td>
<td>Classical seminoma, some lymphocytic infiltration</td>
<td></td>
<td>DD, RT-PCR</td>
<td>22</td>
</tr>
<tr>
<td>SEM2</td>
<td>Classical seminoma</td>
<td></td>
<td>DD, RT-PCR</td>
<td>33</td>
</tr>
<tr>
<td>SEM3</td>
<td>Classical seminoma, some parts intratubular</td>
<td></td>
<td>RT-PCR</td>
<td>28</td>
</tr>
<tr>
<td>SEM4</td>
<td>Classical seminoma, some reduction in spermatids</td>
<td>Prostatic cancer</td>
<td>RT-PCR</td>
<td>74</td>
</tr>
<tr>
<td>SEM5</td>
<td>Classical seminoma, some reduction in spermatids</td>
<td>Prostatic cancer</td>
<td>RT-PCR</td>
<td>88</td>
</tr>
<tr>
<td>SEM6</td>
<td>Normal testicular tissue, some atrophy</td>
<td>Prostatic cancer</td>
<td>RT-PCR</td>
<td>88</td>
</tr>
<tr>
<td>SEM7</td>
<td>Normal testicular tissue, some atrophy</td>
<td>Prostatic cancer</td>
<td>RT-PCR</td>
<td>59</td>
</tr>
</tbody>
</table>

**RT-PCR**

RNA was purified as described above, cDNA was synthesized using a dT<sub>24</sub> primer. Specific primers (~20 bp) targeting each mRNA were designed. PCR was performed in 30 µl of (final concentrations): 12 mmol/l Tris–HCl, pH 8.3; 50 mmol/l KCl; 1.9 mmol/l MgCl<sub>2</sub>; 0.1% Triton X-100; 0.005% gelatine; 250 µmol/l dNTP; 30 pmol of each primer. β-Actin was used as an internal control in all PCR reactions by including 10 pmol of each actin primer (ACCCACACTGTCGGCCATTCA and TCACCTTCACCGTTCCAGTTT) in the reaction, resulting in an ~800 bp actin fragment. Cycle conditions: one cycle of 2 min at 95°C, 30–40 cycles (depending on the intensity of bands) of: 30 s at 95°C, 1 min at 62°C, 1 min at 72°C and finally one cycle of 5 min at 72°C. PCR products were run on 1.5% agarose gels and visualized by ethidium bromide staining. Digital images of the agarose gels were quantified by the STORM phosphor imager software (ImageQuant, Amersham Biosciences). Representative bands from each primer combination were excised, cloned (with TOPO<sup>®</sup> Cloning InVitrogen, Cat. No. 45-0641) and sequenced for verification. In a few of the RT–PCR analyses, no bands were detectable after PCR and nested primers were designed. One micro litre from the first PCR reaction was transferred to a new reaction containing the nested primers and analysed as above.

**Preparation of biotin-labelled probes and ISH**

Probes for ISH were prepared by reamplification of the DDRT–PCR fragments using nested primers specific to the individual fragments with an added T<sub>3</sub>-promotor sequence (AATTAACCTCACTAAAGGG) in combination with the T<sub>7</sub>-extended downstream primer. PCR conditions were: 5 min 95°C; five cycles of: 30 s 95°C, 1 min 45°C, 1 min 72°C; 20 cycles of 30 s 95°C, 1 min 65°C, 1 min 72°C and finally 5 min 72°C. The resulting PCR product was purified on a 2% low melting point agarose gel and sequenced from both ends, using Cy5-labelled primers complementary to the added T<sub>3</sub> and T<sub>7</sub> tags. Aliquots of ~200 ng were used for in vitro transcription labelling, using the MEGAscript-T3 (sense) or MEGAscript-T7 (anti-sense) kits, as described by the manufacturer (Ambion, USA). The composition of the 10× nucleotide mix was: 7.5 mmol/l ATP, GTP and CTP, 3.75 mmol/l UTP, 1.5 mmol/l biotin-labelled UTP. To estimate quantity and labelling efficiencies, aliquots of the labelled RNA product were analysed by agarose gel electrophoresis, and dotted onto nitrocellulose filters and developed as described below.

ISH was performed essentially as described previously (Nielsen et al., 2003). The only deviation from the standard protocol was the removal of mercuric chloride from sections fixed in Sive fixative (Giwercman et al., 1991) by 15 min treatment with potassium iodide (KI) (10 g/l)/I (5 g/l), followed by three washes in diethyl pyrocarbonate (DEPC) water. The iodine was subsequently removed by incubation in sodium thiosulphate pentahydrate (5 w/v, 10 min) followed by washing (4×DEPC water). The ISH procedure in brief: sections were fixed in 4% paraformaldehyde (PFA), treated with proteinase K (P-2308; Sigma, USA) (1.0–5.0 µg/ml), post-fixed in PFA, pre-hybridized 1 h at 50°C, and hybridized overnight at 50°C with biotinylated antisense and sense control probes. Excess probe was removed with 0.1×standard saline citrate (60°C) 3×30 min. Visualization was performed using streptavidin conjugated with alkaline phosphatase (1:1000) (Cat. No. 1093266; Roche Diagnostics GmbH, Germany) followed by development with BCIP = 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/Nitroblue Tetrazolium (NBT).

**Results**

**DDRT–PCR screening**

To obtain knowledge on gene expression changes between the different stages in the development from non-malignant testicular...
tissue through CIS to the malignant stage of a germ cell cancer, we performed a systematic comparison by the DDRT±PCR method (Liang and Pardee, 1992; Jorgensen et al., 1999). For the initial screening, we analysed six testicular biopsy samples: two with homogeneous classical seminoma, two containing almost exclusively CIS tubules, of which one was from an intra-abdominal CIS and two with normal spermatogenesis (Table I). We included the specimen of undescended testis with CIS because according to the hypothesis of testicular dysgenesis syndrome (Skakkebaek et al., 2001), both testicular cancer and undescended testes are symptoms of maldeveloped gonads and may have common pathogenesis.

We performed 402 primer combinations, and assuming that each competitive PCR reaction gives rise to ~100 bands on a DDRT gel and that each band corresponds to one mRNA, this investigation allowed comparison of ~40 000 mRNA. We excised and sequenced 56 bands, which showed overexpression (ranging from 1.5- to 10-fold) in at least one of the CIS samples. Several mRNA were detected with more than one primer combination; for instance, overexpression of \textit{CCND2} was detected with three different primer combinations. Examples of screening gels are shown in Figure 1.

Verification of differential expression

Screening results suggested notable sample-to-sample variation in the expression of many of the bands, even among the normal samples, a phenomenon possibly caused by biological variation and differences in cellularity. To analyse the pattern of expression in detail, all genes showing >2-fold overexpression in CIS were analysed by RT±PCR on a panel of 15 different testicular biopsy samples, including several with seminoma, embryonal carcinoma, mature teratoma, CIS and normal spermatogenesis (Table I). Specific primers targeting each of the putative differentially expressed mRNA were designed, and PCR was performed with \( \beta \)-actin as an internal PCR control and for normalization of PCR and gel loading. As a control, representative DNA fragments from all amplifications were excised, cloned and sequenced.

The RT±PCR analysis verified overexpression of 23 known genes, four transcripts corresponding to EST and one sequence not annotated as a gene (Table II). The overexpression was categorized as either up-regulated in CIS (if the up-regulation was detected in three or four of the analysed CIS samples) or up-regulated in only some CIS samples (if the up-regulation was detected in one or two of the CIS samples). We identified overexpression of a number of genes that have not previously been identified in germ cell neoplasia, which will be discussed below, and genes that have previously been described in CIS or seminoma, such as \textit{CCND2}, lactate dehydrogenase B (\textit{LDHB}) and \textit{XIST} (Houldsworth et al., 1997; Looijenga et al., 1997; Von Eyben et al., 2000; Kawakami et al., 2003). In addition, we identified genes with high up-regulation in the lanes with normal tissue, e.g. protamine 1 and the testis-specific lactate dehydrogenase C. We also detected a weak up-regulation in CIS and tumour specimens of ribosomal proteins L27 and S16, which most likely was related to increased proliferation (Bévort and Leffers, 2000). As an indirect control of the tissue composition, we analysed the expression of a marker of ongoing spermatogenesis, protamine 1, and the mRNA expression of the immunohistochemical marker of CIS, ALPP (Manivel et al., 1987). Our microscopy examination of the biopsies was in good agreement with the expression levels of these markers (Figure 2).

Identification of cell specific expression

To determine whether the detected overexpression was actually caused by expression in CIS cells or was due to expression in other cell types present in the testicular sample, we performed non-radioactive ISH on Stieve-fixed, paraffin-embedded testicular biopsies containing either CIS, seminoma or normal spermatogenesis. Not all ISH probes gave signals possible to interpret, e.g. due to a very low abundance of transcripts or too strong background staining (Table II). To validate

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**Figure 2.** Representative examples of the RT–PCR verification of DDRT–PCR results. (A) Agarose gels displaying RT–PCR analyses. In each panel the top bands are the \( \beta \)-actin control, and the gene name is on the right side of the panel. The last two gels shown are the control analyses, \textit{PROT1} and \textit{ALPP}. (B) Semiquantitative phosphor imager quantification of \textit{CCND2} and \textit{TRA1}. The RT–PCR quantification was done by digitized bit-map image of the agarose result. M = 100 bp marker; SEM = seminoma; TER = teratoma; EC = embryonal carcinoma; CIS = carcinoma \textit{in situ}; NOR = normal testis.
<table>
<thead>
<tr>
<th>Gene identification</th>
<th>Expression</th>
<th>ISH</th>
<th>Locus</th>
<th>Accession no.</th>
<th>References</th>
<th>Possible function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SFRP1</strong></td>
<td>Up in CIS and TER</td>
<td>CIS</td>
<td>8pter-qter</td>
<td>NM_003012</td>
<td>Walsh and Andrews, 2003; Freemantle et al., 2002</td>
<td>Wnt antagonist, inhibition of differentiation, tumour suppressor, is repressed in RA-treated NT2/D1 cells</td>
</tr>
<tr>
<td><strong>TXNIP</strong></td>
<td>Up in CIS and TER</td>
<td>Sertoli</td>
<td>1q11</td>
<td>NM_006472</td>
<td>De Vos et al., 2003; Ikarashi et al., 2002; Trejo et al., 1997; Rasmussen and Means, 1989</td>
<td>Tumour suppressor, negative regulator of thioredoxin</td>
</tr>
<tr>
<td><strong>CALD1</strong></td>
<td>Up in CIS, down in SEM</td>
<td>UA</td>
<td>7q33-7q34</td>
<td>AJ223812</td>
<td></td>
<td>Involved in cell growth and differentiation, high expression accelerates cell cycle progression rendering it a functional oncogene</td>
</tr>
<tr>
<td><strong>CD74</strong></td>
<td>Up in CIS and some SEM</td>
<td>NA</td>
<td>5q31-q33</td>
<td>NM_004355</td>
<td>Badve et al., 2002</td>
<td>Influences expression and peptide loading of MHC II molecules</td>
</tr>
<tr>
<td><strong>C7</strong></td>
<td>Up in CIS, down in all tumours</td>
<td>UA</td>
<td>5p13.1</td>
<td>BC025402</td>
<td>Podack et al., 1984</td>
<td>Part of the humoral immune defence system against invading microorganisms</td>
</tr>
<tr>
<td><strong>CXCL16</strong></td>
<td>Up in CIS, some SEM and EC</td>
<td>NA</td>
<td>17p13</td>
<td>NM_022059</td>
<td>Shimaoka et al., 2000</td>
<td>Chemokine/scavenger receptor that attracts activated lymphocytes during inflammation</td>
</tr>
<tr>
<td><strong>CYBRD1</strong></td>
<td>Up in CIS</td>
<td>CIS</td>
<td>2q31</td>
<td>NM_024843</td>
<td>McKie et al., 2001</td>
<td>Involved in mitochondrial respiratory chain</td>
</tr>
<tr>
<td><strong>CCND2</strong></td>
<td>Up in CIS and all tumours</td>
<td>CIS</td>
<td>12p13</td>
<td>NM_001759</td>
<td>Bartkova et al., 2003; Houldsworth et al., 1997</td>
<td>Involved in RB controlled checkpoint, overexpressed in TGCT</td>
</tr>
<tr>
<td><strong>SALL1</strong></td>
<td>Up in some CIS and up in tumours</td>
<td>U.A.</td>
<td>16q12.1</td>
<td>Y18265</td>
<td>Ma et al., 2002; Kiefer et al., 2002</td>
<td>Represses transcription by recruiting a histone deacetylase complex</td>
</tr>
<tr>
<td><strong>TRA1</strong></td>
<td>Up in some CIS and all tumours</td>
<td>CIS</td>
<td>12q24.2-q24.3</td>
<td>NM_003299</td>
<td>Li et al., 2002</td>
<td>Maintains homeostasis in secretory pathways, involved in MHC presentation/integrin function</td>
</tr>
<tr>
<td><strong>COL1A1</strong></td>
<td>Up in some CIS and TER, down in SEM</td>
<td>CIS and Sertoli</td>
<td>7q21.3-q22.1</td>
<td>BC054498</td>
<td>Prockop et al., 1995</td>
<td>Subunit of collagen, part of the extracellular matrix</td>
</tr>
<tr>
<td><strong>HIST2</strong></td>
<td>Up in some CIS, Some SEM and TER</td>
<td>U.A.</td>
<td>5q31.1-q31.2</td>
<td>AF059650</td>
<td>Yang et al., 1997</td>
<td>Transcriptional co-repressor, involved in cell cycle progression</td>
</tr>
<tr>
<td><strong>HLA-DRA</strong></td>
<td>Up in some CIS, some SEM and TER</td>
<td>NA</td>
<td>6p21.3</td>
<td>BC032350</td>
<td></td>
<td>Involved in antigen presentation</td>
</tr>
<tr>
<td><strong>OPA1</strong></td>
<td>Up in some CIS, TER and EC</td>
<td>NA</td>
<td>3q29</td>
<td>NM_015560</td>
<td>Delell et al., 2002</td>
<td>Component of hemidesmosomes, expression inversely related to prostate cancer progression</td>
</tr>
<tr>
<td><strong>BPAG1</strong></td>
<td>Up in some CIS and up in SEM</td>
<td>UA</td>
<td>6p12-p11</td>
<td>NM_020388</td>
<td>Vanaja et al., 2003</td>
<td>Proteoglycan that inhibits TGF-β and PDGF activity, part of a feedback system regulating cell growth</td>
</tr>
<tr>
<td><strong>DCN</strong></td>
<td>Up in some CIS, down in tumours</td>
<td>NA</td>
<td>12q23</td>
<td>NM_133507</td>
<td>Ungeforo et al., 1995; Nilli et al., 2003</td>
<td>Involved in cell growth and survival during proliferation, overexpressed in TGCT</td>
</tr>
<tr>
<td><strong>LDHB</strong></td>
<td>Up in some CIS, some SEM and EC</td>
<td>UA</td>
<td>12p12.2-p12.1</td>
<td>NM_002300</td>
<td>Von Eynen et al., 2000</td>
<td>Involved in the inactivation process of female X chromosomes, potential oncogenic implications of X chromosomal gain in TGCT</td>
</tr>
<tr>
<td><strong>XIST</strong></td>
<td>Up in some CIS and all SEM</td>
<td>UA</td>
<td>Xq13.2</td>
<td>M97168</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table II Continued

| Genes and transcripts overexpressed in testes containing carcinoma in situ (CIS) samples are listed. Regulation has been described for tumours, and categorized as ‘up in CIS’ if three or four out of four samples were >2-fold up-regulated in normal compared to CIS samples. If only one or two of the four samples were >2-fold up-regulated, this was categorized as ‘up in some CIS’. If up-regulation was less than doubled, it was categorized as ‘weak up in CIS’. The newly identified overexpressed transcripts can be grouped by their ontology into mRNA involved in cell growth/proliferation, cell differentiation, immunological response, and genes with unknown biological function. We discuss a possible significance of the high expression in CIS-containing tissue of selected genes that are interesting from the biological and clinical point of view.

**Discussion**

This is the first systematic study of differential gene expression in testes with CIS, the preinvasive stage of germ cell tumours, compared to normal testis. We have detected genes that were previously reported in CIS and a number of genes never before described in germ cell neoplasia. The newly identified overexpressed transcripts can be grouped by their ontology into mRNA involved in cell growth/proliferation, cell differentiation, immunological response, and genes with unknown biological function. We discuss a possible significance of the high expression in CIS-containing tissue of selected genes that are interesting from the biological and clinical point of view.

**Genes related to cell growth/differentiation**

Several of the overexpressed genes were reported as oncogenes or tumour suppressors in other types of human neoplasms, and may be involved in the neoplastic transformation of germ cells. Among the genes in this category is Frizzled related protein 1 (SFRP1) which was overexpressed in all CIS samples and in the teratoma sample. The transcript was localized by ISH to CIS cells. SFRP1 is a soluble Wnt antagonist, and has been suggested as a tumour suppressor (Bafico et al., 1999). Wnt activation promotes development of many organs, including neural differentiation (Cadinag and Nusse, 1997). Inhibition of Wnt and activation of Notch are features of undifferentiated human embryonal carcinoma (EC) and embryonic stem (ES) cells (Walsh and Andrews, 2003), and may be mediated by SFRP1, which is repressed in differentiating NT2/D1 cells (derived from human EC cells) after treatment with retinoic acid (Freemantle et al., 2002). Increased **SFRP1** expression has been reported in leiomyomas, particularly under estrogenic stimulation (Fukuhara et al., 2002). In this context it is noteworthy that a relative excess of estrogens during early fetal life was proposed as a possible factor involved in the malignant
transformation of germ cells into CIS cells (Sharpe and Skakkebaek, 1993). In addition, changes of methylation status of *SFRP1* were observed in human cancers (Suzuki et al., 2002). Thus, a high expression of *SFRP1* in CIS cells may be also explained by their phenotypic similarity to primordial germ cells, which undergo global demethylation (Van Gurp et al., 1994).

*IGFBP6* was overexpressed in some CIS and the teratoma sample, and the transcript was localized to CIS cells. This protein has preferential affinity for IGFII, which is considered of primary importance for fetal growth. IGFBP6 inhibits cell growth through IGF-dependent and -independent mechanisms, and is a potent inducer of programmed cell death in lung cancer cells (Sueoka et al., 2000). *IGFBP6* expression was previously detected in four of 13 samples of CIS by immunohistochemistry (Drescher et al., 1997). The same study also reported the presence of insulin-like growth factor-II (IGF-II) in Sertoli cells, therefore it is possible that the IGF-II/IGFBP6 system is involved in signalling of growth inhibitory signals from Sertoli cells to germ cells.

Histone deacetylase 3 (*HDAC3*) was overexpressed in one CIS, one seminoma and one teratoma sample. It is a transcriptional co-repressor that plays a role in transcriptional regulation, cell cycle progression and development (Yang et al., 1997). *HDAC3* siRNA caused histone

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**Figure 3.** Identification of the expressing cell types by in situ hybridization (ISH). ISH was performed with anti-sense and control sense (inserted images) RNA probes. Expressing cell types are indicated by arrows. (A) *IGFBP6*, expressed in carcinoma in situ (CIS) cells. (B) *COL1A2*, expressed in CIS cells and Sertoli cells. (C) *SFRP1* expressed in CIS cells. (D) *CCND2* expressed in CIS cells. (E) *TXNIP*, expressed weakly in Sertoli cells in a tubule with CIS cells, the apparent expression in Leydig cells is an artefact, which was also observed for the sense control. (F) *CD47*, expressed in Leydig cells surrounding a tubule with CIS cells; weaker expression is also seen in the Sertoli cells, but not in CIS cells. (G) *ALPP*, included as an ISH control, since *ALPP* is strongly expressed in CIS (Manivel et al., 1987). (H) Protamine, included as an ISH control for normal tubules, as protamine is expressed in spermatids. Scale bar = 50 μm.
hyperacetylation and increased apoptosis, demonstrating that HDAC3 is important for survival of cancer cells (Glaser et al., 2003). Molecular partners (co-repressors) and target genes for HDAC3 in the testis are unknown, although it is possible that two other over-expressed genes, TTNIP and SALL1, may be involved. Thioredoxin interacting protein (TTNIP, also known as VDUP1) was weakly overexpressed in all CIS and the teratoma sample, and by ISH the transcript was localized to Sertoli cells in tubules with CIS. TTNIP has tumour suppressor activity, interacts with histone deacetylase 1 and is a negative regulator of thioredoxin, which is a redox protein reported to function as an inhibitor of the cell cycle and a stimulator of apoptosis (Nishiyama et al., 1999; Han et al., 2003). Down-regulation of TTNIP has been reported in lymphoma and gastric cancer (Ikarashi et al., 2002; De Vos et al., 2003). The Zinc finger protein SALL1 was overexpressed in one sample with CIS and in all tumour samples. It is expressed in fetal and adult Leydig and Sertoli cells (Ma et al., 2002). Murine Sall1 represses transcription by recruiting a histone deacetylase complex (Kiefer et al., 2002).

Decorin (DCN), overexpressed in some CIS samples and down-regulated in the tumour samples, is a proteoglycan that in adult testis was detected in fibroblasts, peritubular cells and arteries but not within seminiferous tubules (Ungefroren et al., 1995). DCN inhibits TGF-β and PDGF activity, thus part of a feedback system regulating cell growth (Yamaguchi et al., 1990; Nili et al., 2003). Interestingly, DCN was identified in certain stages of the migratory pathway of primordial germ cell in the mouse embryo (Soto-Suazo et al., 2002), and was also required to reverse the migratory phenotype of myoblasts in the developing chick limb buds (Oguin et al., 2003). Therefore, we speculate that the enhanced expression of DCN in tissue containing CIS cells may reflect their primordial germ cell-like phenotype.

**Genes related to the immune system**

Several of the genes overexpressed in specimens with CIS are related to an activated immune system, which corresponds well to the frequent presence of mononuclear cell infiltration in tissues with CIS and seminoma (Jahnukainen et al., 1995). The detected up-regulation of these genes is therefore presumably not derived from CIS cells, but is more likely caused by infiltrating lymphocytes or other cells involved in the immune response. Tumour rejection antigen 1 (TRA1) was up-regulated in one CIS and all tumour samples investigated; it is ubiquitously expressed and is a peptide binding protein in the endoplasmic reticulum implicated in integrin function and host defence action (Li et al., 2002). Other up-regulated genes found in this study that are related to the immune system are CD47, CD74, complement component-7, SRP-SOX chemokine, interferon-induced transmembrane protein-1 and HLA DR alpha chain.

**Other differentially expressed genes**

Up-regulation of collagen alpha 2 type 1 (COLIA2) and bullous pemphigoid antigen 1 (BPAG1) is most likely related to remodelling in CIS-containing tissue due to an enhancement of the attachment capacity of the neoplastic cells. In prostate cancer, the expression of BPAG1 was inversely correlated with tumour progression (Vanaja et al., 2003). Two additional up-regulated genes, optic atrophy 1 (OPA1) and cytochrome b reductase 1 (CYBRD1), are probably related to mitochondrial processes, reflecting a changed activity in the mitochondria or changes in number of mitochondria.

Several of the detected genes were only overexpressed in a subset of the CIS samples, which may partly be due to specific aspects of the biology of the testis and CIS cells. First, in frozen tissue specimens it is not possible to determine the exact composition of the analysed testicular tissue by histology of adjacent tissue fragments. On the transcript level, a strong overexpression in one cell type may mask a weak overexpression in the cell of interest. We have partially obviated this problem by ISH analysis of some of the newly identified transcripts. Secondly, morphologically similar-appearing CIS cells in the vicinity of different types of TGCT may have different gene expression profiles. In fact, heterogeneity of CIS cells at the protein level was noticed earlier (Raajtart-De Meyts et al., 1996). Thirdly, the expression profile in the testis seems to be affected by the location of the testsis. Perhaps these gene expression differences are linked to the causative factors involved in the pathogenesis of cryptorchidism, or perhaps the gene expression differences occur as a result of the different location, maybe due to changes in temperature. This may be the reason for the differences in gene expression profile between the sample from an undescended, intra-abdominal testsis (CIS2, Table I) and other CIS samples. For example, TRA1 was relatively weakly expressed in CIS2, whereas HDAC3 was strongly overexpressed. One sequenced band (novel EST: OIC1 (Overexpressed In CIS), Table II), which was strongly overexpressed in CIS2, did not match any mRNA or EST sequence in the genbank or EBI databases. However, OIC1 had a perfect match to a genomic sequence located on chromosome 15q21.3, within the large second intron in the gene for TCF12 (also called HTF-4 and E-Box binding protein). The match was downstream of an EST that is also located in the second intron of TCF12 and is transcribed independently of TCF12 (accession no. BX112789, AI075936, Unigene cluster Hs.134007). To investigate whether the sequenced band was derived from alternative splicing of either TCF12 or the EST, we designed primers matching exon 1 and 2 of TCF12 and exon 1 and 2 of the EST and performed PCR with a reverse primer located at the 5′-end of the sequenced fragment. None of the primer combinations produced a band after PCR, suggesting that the detected transcript may correspond to a novel mRNA. The sequence has been submitted to the EBI database with accession no. AJ617288.

The results of our search for differentially expressed genes in CIS demonstrated a number of genes linked to testicular development, i.e. DCN, IGFBP6, SFRP1, SALL1, supporting our hypothesis of the origin of CIS from primordial germ cells or gonocytes (Skakkebaek et al., 1987). In regard to the future, the results of this study indicate that differences between the gene expression profiles in CIS compared to normal tissue are informative with regard to the biology and origin of this neoplasia. More work is needed to describe the transcriptome of CIS cells in detail. A comparison between CIS and the cell type from which CIS derives (fetal germ cells) would be very informative for the elucidation of mechanisms of neoplastic transformation, but this remains practically very difficult due to problems in obtaining human fetal gonocytes. The other important perspective is to apply the new knowledge for early clinical diagnosis of germ cell neoplasms at the stage of CIS, possibly by a non-invasive method. Further studies to define the biological importance of overexpression in CIS of the newly identified genes and their possible use for early detection of incipient testicular cancer in the clinical setting are planned.

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