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Research Article

Gene signatures of testicular seminoma with emphasis on expression of ets variant gene 4

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Abstract. Gene expression patterns of testicular seminoma were analysed applying oligonucleotide microarrays in 40 specimens of different tumour stages (pT1, pT2, pT3) and in normal testes. Transcripts of maternally expressed 3 transcripts were expressed in seminoma without correlation with delta-like 1 homologue expression indicating an impaired imprinting status in seminoma. Interestingly, the transcripts of bromodomain-containing 2 and nuclear autoantigenic sperm protein associated with spermatogenesis were significantly upregulated in progressing tumour stages. Transcription factors TEA domain family member 4 and ETS variant gene 4 (ETV4), weakly expressed in normal testis, were strongly augmented during tumourigenesis. For ETV4 expression, a significant correlation with the increased expression of matrix metalloproteinase 2 and a disintegrin and metalloproteinase domain 15 was determined. The ETV4 protein was localised to nuclei of spermatogonia and revealed an intense staining in seminoma cells. Taken together, we characterised additional transcription factors and spermatogenesis-associated genes involved in the progression of seminoma.

Key words. Germ cell tumours; pure seminoma; ETV4 (PEA3, E1AF); TEAD4 (TEF3, RTEF1); MEG3 (GTL2); DLK1 (FA1); BRD2; NASP; gelatinase A; MMP2; metargidin, ADAM15.

Human testicular germ cell tumours derive mostly from the primordial germ cell lineage. Although testicular germ cell tumours are rare malignancies (1–3 %), they are the most common solid tumour among men between 15 and 45 years [1]. Testicular germ cell tumours are classified into two main histological subtypes, seminoma and non-seminoma.

In the United States, the incidence of testicular germ cell tumours showed an increase of 44 % between 1973 and 1998 [2]. The worldwide incidence increase of seminoma was approximately 64 %. Despite its increasing

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incidence, only a few risk factors are known, including cryptorchidisms and family clustering of testicular cancer [3, 4]. Environmental factors and nutrition such as the increasing presence of oestrogenic compounds have been suggested to play a role [5]; however, epidemiological studies have shown contradictory results [6, 7]. Besides those risk factors, reliable prognostic markers are not known. Elevation of human chorionic gonadotropin (hCG) [8] or fetoprotein [9] do not represent a special entity of carcinoma and are inconsistent in their expression levels.

In recent years, several genes and chromosomal aberrations have been identified which are considered to be involved in the pathogenesis of and predisposition for testicular germ cells tumour [10, 11]. The most consistent chromosomal aberration in testicular germ line tumours is the presence of an isochromosome of the short arm of chromosome 12 as reviewed previously [12]. Expression of two genes located in this region, the proto-oncogene K-RAS2 and cyclin D2 (CCND2), were enhanced in the tumours; however, whether the presence or the amplification is of prognostic relevance remains to be proven [12].

Unlike non-seminoma, seminoma consists of relatively uniform cell populations associated with lymphocyte infiltrates [13]. Though these tumours are characterised by a homogeneous histology, differences in therapeutic outcome may indicate that seminoma is comprised of different tumour entities, probably due to differences in gene expression patterns.

To evaluate clinical markers and to gain more insight into the mechanisms of tumorigenesis, we applied oligonucleotide microarrays to further characterise gene expression signatures which may be involved in tumour progression and could represent candidate genes for prognosis and diagnosis.

Materials and methods

Tissue samples. Seventy-two testicular tissue samples were employed in this study for gene array and RT-PCR analyses, including nine normal testes (NT, three autopsy cases of men with normal spermatogenesis, three tissues from orchiectomy cases due to prostate carcinoma, three biopsies with status after vasectomy). All patients underwent surgery between 1995 and 2003 at the Departments of Urology at the University of Duisburg-Essen in Essen, the University Hospital in Mannheim and the University of Muenster (Germany). Their age ranged from 21 to 58 (mean 38). Histological diagnosis for pure seminoma and evaluation of the tumour stages were performed on representative tissue sections by conventional light microscopy as proposed by the American Joint Committee on Cancer and the International Union against Cancer [14]. The distribution of the different stages of pure seminoma was: stage pT1, n = 40; pT2, n = 19; pT3, n = 4. All specimens were collected after obtaining the informed consent from the patients (clinical data are listed in supplementary data, see table S1, http://www.uni-essen.de/anatomie/contents/agwiha/dokumente/seminoma_supp.pdf).

RNA preparation and cDNA synthesis. Tissues were lysed in TRIzol (Invitrogen, Karlsruhe, Germany) and total cellular RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA quality was controlled by gel electrophoresis and spectrophotometrically. Samples of high-quality RNA were further processed and hybridised to a HG-U95Av2 array (Affymetrix, Santa Clara, Calif.).

Microarray analyses. Preparation of cDNA targets starting from 5 to 10 μg of total RNA, fragmentation, hybridisation to HG-U95Av2 arrays, washing, staining and scanning were performed following manufacturer protocols. Expression values (Signal and Detection calls: A – absent, P – present, M – marginal) were obtained by performing the absolute analysis using Affymetrix microarray Suite Version 5.0. This process includes the background correction of the average of the lowest two percentiles of intensities on a 4×4 grid on the chip, the introduction of an 'ideal mismatch' forced to be lower than the corresponding perfect match, and the use of Tukey's biweight to elicit an expression value out of single-probe intensity pairs.

Analyses of arrays of 40 seminoma and three normal testicular samples showed 3'/5' ratios of <3.0 for the β-actin control, indicating good RNA quality and cDNA synthesis, and these were selected for further data processing. Global scaling was applied to allow comparison of gene signals across multiple microarrays. Annotation of the probe sets was taken from the annotation files provided on the Affymetrix homepage [15].

Hierarchical clustering analysis. Hierarchical clustering analysis was performed using 1017 genes called present (P) in at least 3 of 43 samples (fig. 1A). Signals were normalised to the median signal of all analyses and logarithmically transformed (base 10). We selected genes with a standard deviation (SD) ≥ 0.4 of the normalised data. Genes and samples were grouped by hierarchical clustering (Spotfire, Somerville, Mass.) using the unweighted average (UPGMA) method and the Euclidean distance to measure the similarity. The average value was used as an ordering function to generate the clusters. In the second hierarchical clustering analysis performed in a similar manner and presented in figure 1B, we included 40 seminoma samples and 138 genes expressed in at least 30 specimens (75 %) with an SD ≥ 0.4 of the normalised data.

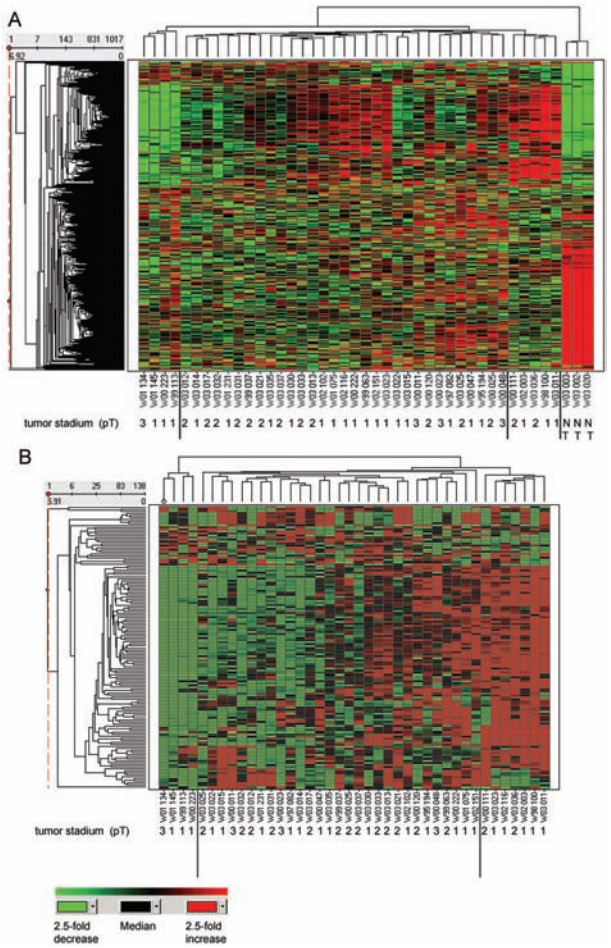

Figure 1. (A) Hierarchical clustering of 1017 differentially expressed genes in 40 seminoma samples of three different tumour stages (pT1–pT3), and in three normal testicular specimens (NT). Genes which are present in at least three tissue samples (p-call > 3) with a fold change of ≥ 2.5 compared to the median expression are included. (B) Hierarchical clustering of 138 differentially expressed genes in 40 seminoma samples. Genes which are present in at least 30 tissue samples (>75 %) with a fold change of ≥ 2.5 compared to the median expression are shown. For normalisation, expression values were divided by the median expression value of the respective gene and logarithm transformed. The color-coded scale for the normalised expression values ranged from green (2.5-fold decrease) to red (2.5-fold increase) with a black code for the median value.

Semiquantitative RT-PCR. Semiquantitative competitive RT-PCRs were performed for a total of 25 samples analysed before by microarrays and for an additional 10 tissues of another cohort of patients (2 × NT, 5 × pT1 and 3 × pT2) by standard techniques using 2 µg of total cellular RNA and oligo dT priming. Primer sequences specific for human TEAD4 (annealing at 60 °C/number of PCR cycles: 30/25); ETV4 (65 °C/30 and 26 cycles); MMP2 (60 °C/28 cycles); MMP12 (60 °C/28 cycles); MMP15 (60 °C/30 cycles); ADAM15 (60 °C/30 cycles); DLK1 (60 °C/30 cycles) and MEG3 (60 °C/30 cycles) are available in supplementary data (table S2). Since most of the frequently used housekeeping genes were differentially expressed in seminoma [16] we chose the UBB gene (ubiquitin B), which was not regulated in seminoma, and co-amplified it as internal standard control in each experiment. The densitometric analyses applied the Scion Image software (Scion Corporation, Frederick, M.).

Immunohistochemical analysis. Formalin-fixed, paraffin-embedded specimens from patients with seminoma were randomly selected from the group described above. The sections were subjected to antigen retrieval by immersing them in 0.01 M sodium citrate (pH 6.0) and heating in a microwave oven. The subsequent immunohistochemical staining for ETV4 (200 µg/µl of monoclonal murine antibody sc-113; Santa Cruz Biotechnology, Santa Cruz, Calif.) was performed applying the DAB method using the mouse Vectastain kit (Vector Laboratories, Burlingame, Calif.) and DAB substrate (DakoCytomation, Hamburg, Germany) according to manufacturer protocols, followed by counterstaining with haemalaun.

Statistical analysis. Exploratory data analysis, the non-parametric Kruskal-Wallis test as the Mann-Whitney test for the non-parametric independent two-group comparisons and Pearson's correlation were performed with the program SPSS 10 for Windows (SPSS, Chicago, Ill.). Differences with $p \leq 0.05$ were regarded as statistically significant.

Results

Gene expression analyses of seminoma tissues. A total of 40 pure seminoma including 22 samples of tumour stage 1 (pT1), 14 samples of tumour stage 2 (pT2) and 4 of tumour stage 3 (pT3) as well as three normal testicular samples of high-quality mRNA were used for microarray analyses.

The hierarchical cluster analysis clearly separated the 40 seminoma specimens from the three normal testis specimens (fig. 1A). The seminoma samples showed a clear division into three main subclusters. As indicated in figure 1A, these subclusters did not correspond to the histologically determined tumour grade.

To elucidate the genes separating the seminoma into subclusters, a further hierarchical cluster analysis of the seminoma specimens was performed (fig. 1B). All genes included were expressed in at least 30 out of 40 seminoma specimens (75 %) and their normalised expression ratios varied on average at least 2.5-fold from the median. The expression patterns of 138 differentially expressed genes differentiated the seminoma population into three distinct groups with 4, 29 and 7 samples, respectively. There was no clear separation between the different tu-

Table 1. Two hundred and thirty-one genes upregulated at least 4-fold in mean expression from normal testicular tissue to seminoma stage pT1, according to their biological function.

Biological Process	Gene Symbols	Factor of Increase, Range
Cell motility	ARPC1B, MSN, PFN1, TPM1	4.8 to 8.2
Protein metabolism	CTSH, GZMK, LOXL2, PRODH, PSMB9, SLC7A6, UBD, VAMP8	4.5 to 115.5
Energy metabolism	ACP5, CD38, CHIT1, CYBB, FAM16AX, FBP1, GLDC, MTHFD1, NAGA, PHKA1, PYGL, SOAT2, UGCG	4.8 to 33.6
Lipid metabolism	APOC1, APOE, CD2, LIPA, OSBPL3, PLCG2	4.1 to 24.5
Proliferation	ACVR1, AK3, APEX1, AURKB, BASP1, BTG2, C2F, CDX1, CDC25B, CENPA, CCND2, CCNF, CRIP1, DOCK4, EVI2B, ECGF1, ETV1, ETV4, FKBP1B, FYB, GMFG, HCLS1, HHEX, HCK, HIST1H1C, IFITM1, LMNB2, LRRFIP1, LCK, MDK, MAP4K1, MSC, NRF, NME2, NFE2L3, NFKB2, NP, PIM1, PIM2, PQBP1, POU2AF1, POU5F1, RECQL, RNASE6, RPL10A, RRS1, STAT1, SOX13, SOX4, GG2-1, TFAP2C, UPP1, VENTX2, ERBB3, MYBL2, MYCN, LYN, ZNF124, ZFP36L2	4.0 to 134.4
Negative regulation of cell proliferation	BAX, CFLAR, CASP1, CASP4, CASP8, DLG3, GPNMB, GZMA, GZMB, GZMH, HIC2, MSH2, MX1, REA, RARRES3, TRAF1, TNFSF10, TNFRSF25, TNFRSF7	4.0 to 59.6
Transport	AQP3, ABCC2, CACNB3, FTL, GLRX, KCNJ8, SLC1A3, SLC25A6, SLC25A16, SLCO2B1, TXN, THOC2, MGC8685	4.0 to 16.9
Signal transduction	ADAM19, ADAM8, ADAMDEC1, ADCY7, CDH3, CD53, CHN2, CLECSF2, EFEMP1, GIP, GPX2, GRN, INPP5D, ITGAX, ITGB2, JUP, MMP12, MMP9, PIK3C2B, PLEK, PTPN12, PTPRC, RAB7, RAB7L1, RAC2, RGS10, RGS19, SELL, SLA, TGFBI, TRO	4.0 to 147.3
Immune response	AIF1, B2M, OK/SW-cl.56, CAPG, CD14, CD3D, CD3Z, CD74, CCL2, CCL4, CCL5, CCR1, CCRL2, CXCL10, CXCL9, CXCR4, F2RL1, CSF2RB, C1QB, C2, CST7, FCER1G, FCGR2A, FCGR3A, GBP1, IGHG3, IGJ, IGSF6, INDO, G1P2, IFI30, IL2RG, LILRB1, LILRB4, LY75, LY96, LCP2, LTB, HLA-C, HLA-E, HLA-F, HLA-F, HLA-DMA, HLA-DMB, HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRB3, MICB, NK4, NCF4, PDLIM1, PLA2G7, PLAU, RGS1, ARHGDIB, SAMHD1, SERPINA1, SIAT1, TMSB4X	4.4 to 54.2
Development	ALPL, DSP, GSTP1, GYPC, IL6R, LMO2, MDFI, NRCAM, PLOD, PROM1, RPS6KA3, SPP1, SOX15, TCL1A, TCL1B, TEAD4	4.5 to 79.0

A further 120 upregulated genes or ESTs were with unknown function.

mour stages based on the altered gene expression pattern of a large set of genes. The major red cluster of genes shown in figure 1B was responsible for the formation of the three groups and includes genes mainly involved in the immune response, such as immune globulin chains or diverse chemokines (data not shown). No other clear gene cluster specific for particular seminoma subgroups was detected.

Identification of genes characteristic for seminoma. To identify additional genes representative for seminoma, the gene expression profiles of four representative samples of tumour stage pT1 were compared to those of the three normal testicular tissue samples resulting in 12 single comparisons. The expression of 1490 transcripts was determined as differentially expressed in at least 80 % of the comparisons with a change p value < 0.001 and > 0.999 for at least 2.5-fold increased (635 transcripts) and decreased (855 genes) changes (pT1 versus NT), respectively. Upregulated genes belonging to the immune response cascades, such as CCL or HLA (table 1), can be traced back to the leukocytic infiltration of the tumour tissue.

The expression of significantly regulated genes related to progressing tumour stages was evaluated in all 40 seminoma samples. Upregulation of several genes already described as characteristic for seminomas could be confirmed using this approach. We found a 9.9-fold upregulation of CCND2 in seminoma (table 1) [17]. Furthermore, expression of KIT was found in 30 out of 40 seminoma specimens and in 1 out of 3 tissues with normal spermatogenesis according to already published

Table 2. The ten most downregulated genes in 40 seminoma specimens compared to normal testicular tissue, expressed in at least 80 % of the samples.

Probe Set ID	Gene Title	Gene	Gene	P-call	Factor	NT		pT1		pT2		pT3	
		Symbol	Location	Tumour	D	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD
36925_at	heat shock 70kDa protein 2	HSPA2	14q24.1	34	44.3	**16384**	774	**391**	183	**368**	165	**309**	263
40142_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 24	DDX24	14q32	35	29.0	**6746**	511	**232**	100	**224**	68	**294**	73
41071_at	serine protease inhibitor, Kazal type 2 (acrosin-trypsin inhibitor)	SPINK2	4q12	39	28.9	**28905**	1226	**890**	610	**1244**	615	**814**	359
33139_s_at	myeloid leukemia factor 1	MLF1	3q25.1	33	24.6	**16377**	1208	**675**	482	**697**	292	**513**	528
35233_r_at	centrin, EF-hand protein, 3	CETN3	5q14.3	33	18.8	**6167**	1314	**343**	115	**325**	83	**299**	133
35614_at	transcription factor-like 5 (basic helix-loop-helix)	TCFL5	20q13.3	39	16.8	**16387**	598	**989**	442	**1052**	405	**770**	686
37402_at	ribonuclease, RNase A family, 1	RNASE1	14q11.2	35	16.3	**17150**	5373	**1273**	1432	**721**	547	**1103**	631
36780_at	clusterin	CLU	8p21-p12	40	14.9	**59322**	14419	**5423**	5823	**2546**	1473	**1607**	408
1599_at	cyclin-dependent kinase inhibitor 3	CDKN3	14q22	39	14.3	**14279**	1534	**1052**	409	**886**	308	**1023**	515
36606_at	carboxypeptidase E	CPE	4q32.3	37	13.0	**9949**	766	**874**	535	**718**	512	**389**	470

Expression of listed genes was decreased (D) in all comparisons.

data [18]. TCL1 expression, which has already been associated with seminoma [19], was detected as both isoforms, TCL1A and TCL1B, and was significantly upregulated (table 1). However, these transcripts were not further regulated during progressing tumour stages in seminoma.

Genes involved in tumour progression. The majority of the 855 downregulated genes in tumour stage pT1 compared to testicular tissue are involved in pathways necessary for regular spermatogenesis. From the top ten (table 2), the downregulation of the clusterin gene CLU (M25915) and the carboxypeptidase E gene CPE (X51405) was directly proportional to the tumour stage with a maximal 37- and 26-fold decrease, respectively, in pT3 when compared to testicular tissue with regular spermatogenesis. Clusterin has been associated with Sertoli cells, luminal sperm, proacrosomal Golgi complexes, residual bodies and degenerating germ cells [20]. CPE processes prohormone intermediates and CPE-deficient mice showed abnormal testis morphology and function [21], indicating a role in spermatogenesis. More information about downregulated genes is present in supplementary data (table S3).

Focussing on upregulated genes, we compared all seminoma tumour entities staged for pT2 or pT3 (n = 18) with all seminoma tissues from pT1 (n = 22, table 3). Out of 113 upregulated transcripts (83 genes), 50 genes showed a significant increase in expression only from stage pT1 to higher grades. Of the upregulated genes 45 % participate in processes involved in cell cycle control, such as the splicing factor SFRS4 [22], the BOP1 gene encoding a nucleolar protein involved in ribosome biogenesis [23] and the CHC1 gene (RCC1) involved in chromosome condensation [24]. In the following experiments, we focussed on genes which showed a high and stage-dependent regulation in the majority of seminoma tissues tested and which had not yet been described for seminoma.

One of the strongest upregulated genes in seminoma (table 1) was the ETV4/PEA3/E1AF gene (D12765), encoding an Ets-related transcription factor binding to the adenovirus E1A enhancer, and which showed a mean increase factor of 37.3 ($p < 0.001$). ETV4 was expressed in 36 out of 40 seminoma samples but not in tissues with regular spermatogenesis. The expression of *ETV4* increased with progressing tumour stages, as presented in figure 2A. The results were verified using semiquantitative competitive RT-PCR showing a 16.7-fold increase when compared to normal testicular tissue (fig. 2B). In contrast to data from gene arrays, all samples expressed ETV4 mRNA. The expression of ETV4 relative to UBB increased somewhat from stage pT1 to pT3 ($p = 0.088$) and showed a significant increase from stage pT2 to pT3 ($p = 0.009$). ETV4 showed positive immunolabelling restricted to spermatogonia in normal testicular tissue (fig. 3A). Cells of the carcinoma in situ (fig. 3B) as well as seminoma cells (fig. 3C, D) were strongly positive for ETV4. However, the staining intensity varied between the seminoma cells. Leucocytes infiltrating the seminoma were negative for ETV4 (fig. 3B–D). Taking these results together, only spermatogonia expressed ETV4 in normal testicular tissue and the increased levels of ETV4 in seminoma were further enhanced with progression of the tumour.

Because ETV4 is known to regulate the expression of certain matrix metalloproteinases [25, 26] we correlated

Biological Processes	Gene Symbols	Count P, Range	Factor of Increase, Range
Cell adhesion and motility	**AAMP**, **CDH2**, DDR1, **FSCN1**, JUP, **TROAP**, **TSTA3**	12 to 40	1.2 to 2.2
Protein metabolism	**EEF1E1**, GARS, PSMD13, **PTP4A3**, PTPN2, ST5	27 to 40	1.1 to 1.4
Energy metabolism	**AHCY**, **NTHL1**, PKM2, UNG	28 to 40	1.3 to 1.5
Proliferation	ATIC, BOP1, BRRN1, CBX3, CCNF, **CHC1**, **COPS6**, CP110, DDX39, **DHPS**, **EGF**, ESRRG, EZH2, FZR1, GABPB2, **H1FX**, **HNRPDL**, HSF1, **KIFC1**, **KNTC1**, LSM2, **MTERF**, NCKIPSD, **NR4A1**, PML, POLE, **POLR3F**, **POP7**, PPRC1, **PRCC**, RNUT1, SFN, **SFRS4**, **SMARCC1**, **TAF4**, TPX2, UBE2V1, XRCC5, XRCC5	14 to 40	1.2 to 2.0
Transport	ABCC10, **HCN2**, SLC35A2, **SNX4**	19 to 38	1.4 to 1.8
Negative regulation of cell proliferation	TNFRSF25	40	1.4
Signal transduction	ANGPT1, **ARID1A**, **FRAG1**, H1FX, **NPM3**, OK/SW-cl.56, OR2B6, **STMN1**, TBL3, **TRIP10**, VAMP1	26 to 40	1.2 to 1.8
Spermatogenesis	**BRD2**, **NASP**, ZP3	25 to 40	1.2 to 1.7
Unknown	B1, **HUMAUANTIG**, **MGC16824**, MKRN4, **NOL7**, **NSPC1**, **RNPC1**, TACC2, **TSPY1**	17 to 40	1.2 to 2.3

Table 3. Significantly upregulated genes in 18 seminoma specimens (tumour stage pT2 and pT3) compared to 22 tissues of tumor stage pT1, sorted according to their biological activity.

Genes with unchanged expression from healthy tissue to tumour stage pT1 are indicated in bold. The value factor of increase corresponds to the enhancement of expression in pT2 and pT3 when compared to pT1.

the expression pattern detected by microarray analyses of ETV4 to the expression of several metalloproteinases (MMPs) as well as to that of members of the ADAM family. ETV4 expression correlated significantly (measured by Pearson's correlation coefficient with a two-tailed significance test) with the expression of MMP2 (M55593, r = 0.3128), MMP11 (X57766, r = 0.3641), MMP14 (Z48481, r = 0.4054), MMP15 (D86331, r = 0.5664) and ADAM15 (U41767, r = 0.5063), whereas a negative correlation was found with the downregulated ADAM17 (U69611; r = –0.3670). The upregulation of MMP11, MMP14 and MMP15 as well as the downregulation of ADAM17 in seminoma could not be confirmed by RT-PCR (data not shown), but we verified the upregulation for MMP2 and ADAM15 expression (fig. 2C–E). MMP2 was present in 25 of 40 seminoma samples in microarrays and the expression increased significantly from stage pT1 to pT3, resulting in a 2.2-, 2.9- and 3.4-fold increase in expression for stage pT1, pT2 and pT3, respectively (fig. 2C). Semiquantitative RT-PCR analyses showed that all seminoma samples expressed MMP2 (fig. 2E). Relative mRNA expression of MMP2 was 1.23-fold increased (p = 0.050) in seminoma when compared to normal testicular tissue, and the 1.15-fold increase from stage pT1 (n = 4) to pT2 (n = 4) was statistically significant (p = 0.020). In microarrays, ADAM15 (metargidin) was expressed in all samples and was 1.8-fold upregulated when compared to normal testicular tissue in a stage-dependent manner (p = 0.004; fig. 2D) with a maximal 3.9-fold increase in a seminoma specimen in stage pT3. RT-PCR analyses verified a 2.23-fold increase of ADAM15 expression in seminoma tissues when compared to normal testicular tissue (fig. 2E).

Taken together, ETV4 expression was continuously upregulated with tumour progression and was correlated with the activation of MMP2 and ADAM15.

In addition, microarray analysis revealed an increase in expression of the transcription factor TEAD4 (RTEF1, TEF-3; U63824; fig. 4) with an increase of 12.1-, 12.6- and 20.8-fold for pT1, pT2 and pT3, respectively, compared to normal testicular tissue (fig. 4A). This increase was significant in stage pT3 specimens when compared to stage pT1 and pT2. RT-PCR analyses confirmed these findings and showed an 8.3-fold increase from normal testis to pT1 and a significant increase with progressing tumour stages (fig. 4B).

Testis-specific genes in seminoma. Though all seminomas had been histologically diagnosed as pure seminoma, active testis specific genes could still be found.

Some of the testis-specific genes belong to the imprinting genes. MEG3 and DLK1 are reciprocally imprinted genes on human chromosome 14q32 [27]. The maternally expressed MEG3 allele encodes a non-translated RNA whereas the paternally expressed DLK1 encodes a member of the epidermal growth factor superfamily, delta-like

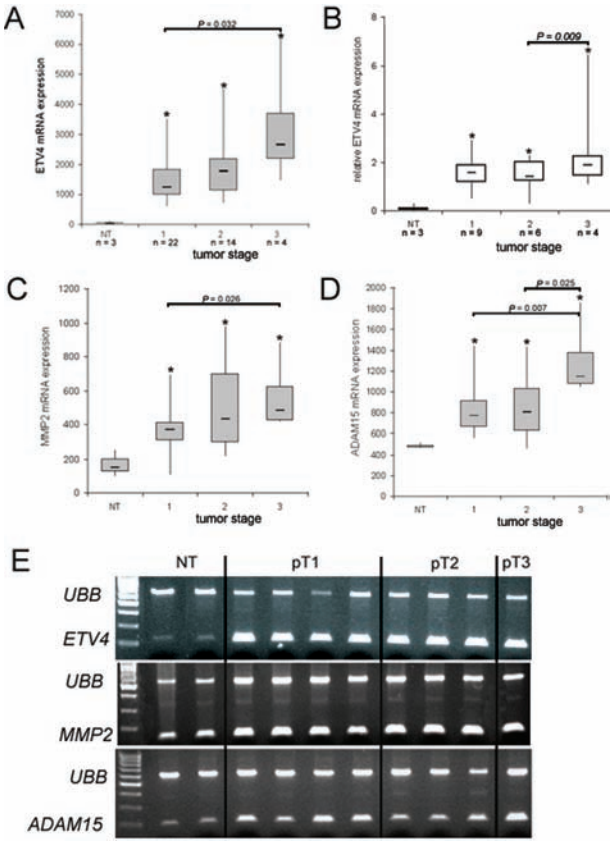

Figure 2. (A) ETV4 mRNA expression in microarrays from 40 seminoma tissues and 3 normal testes. Asterisks mark a significant regulation when compared to normal testicular tissue (NT). The indicated p value denotes a significant difference between the tumour stages pT1 and pT3. (B) Relative expression of ETV4 mRNA analysed by three independent RT-PCRs of 3 healthy testicular tissues and 18 seminoma specimens. Asterisks indicate a significant regulation when compared to normal testicular tissue (NT). (C) MMP2 mRNA expression in microarrays from 40 seminoma and 3 normal testes ($p = 0.012$). Asterisks denote a significant regulation of MMP2 transcription compared to normal testicular tissue (NT). The indicated p value marks a significant difference between the tumour stage pT1 and pT3. (D) Expression of ADAM15 mRNA in microarrays from 40 seminoma tissues and 3 normal testes. Asterisks denote a significant regulation compared to normal testicular tissue (NT). The indicated p values denote significant differences between the tumour stages. (E) Semiquantitative RT-PCRs for ETV4, MMP2 and ADAM15 mRNA expression in normal (n = 2) and seminoma (n = 8) tissues.

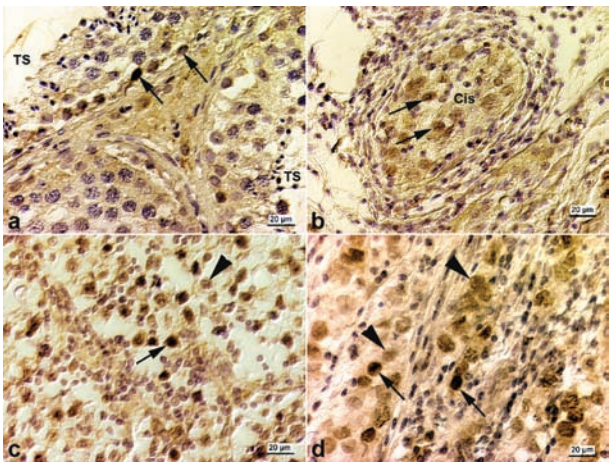

Figure 3. (A–D) Arrows indicate nuclei with strong staining of ETV4; arrowheads mark nuclei with less intense immunostaining. (A) Immunohistochemistry of ETV4 protein in normal testicular tissue in a biopsy of contralateral testis from a seminoma patient, nuclear staining in spermatogonia but no staining in spermatocytes and spermatids. TS, tubuli seminiferi. (B) Immunostaining of ETV4 protein in a carcinoma in situ (Cis) from a seminoma specimen with tumour stage pT1. (C, D) Positive staining for ETV4 in most seminoma cells from specimens with tumour stage pT1 (C) and pT2 (D) but no staining in leucocytic infiltrates.

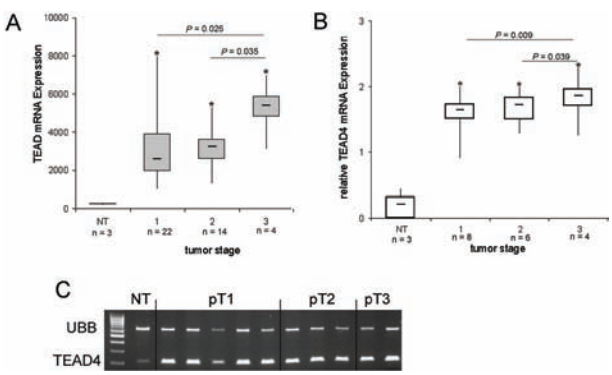

Figure 4. (A) Expression of the transcription factor TEAD4 mRNA in 40 seminoma tissues and 3 normal testes applying microarrays. Asterisks mark a significant regulation when compared to normal testicular tissue (NT). The indicated p values denote significant differences between the tumour stages. (B) Relative expression of TEAD4 mRNA analysed by RT-PCR in 3 normal testicular tissues and 18 seminoma specimens. Asterisks denote a significant regulation when compared to normal testicular tissue (NT). (C) A representative semiquantitative competitive RT-PCR for TEAD4 expression in normal and tumour tissues. The densitometric analysis is presented in B.

(dlk). The microarray analyses showed an upregulation of MEG3 (GTL2, AF052114) in 10 of 12 single comparison analyses with a mean increase of 3.2-fold ($p = 0.005$; fig. 5A). In contrast, all 12 single-comparison analyses for DLK1 (FA1, U15979) revealed a 6.7-fold downregulation ($p = 0.002$; fig. 5A) in 40 seminoma specimens compared to NT. The RT-PCR analyses in 11 samples (fig. 5B, C) showed no regulation of MEG3. DLK1 expression was highly decreased in five seminoma specimens but remained unchanged in four of the tumour tissues. Since the DLK1 protein is known to be expressed in Leydig cells [28], we compared signal intensities of DLK1 and the Leydig-cell-specific gene INSL3 in a microarray and found a strong correlation ($r = 0.845$, $p < 0.0001$), indicating that both genes are Leydig cell specific. The gene encoding the follicle-stimulating hormone receptor, FSHR (M65085), is specific for Sertoli cells and was still

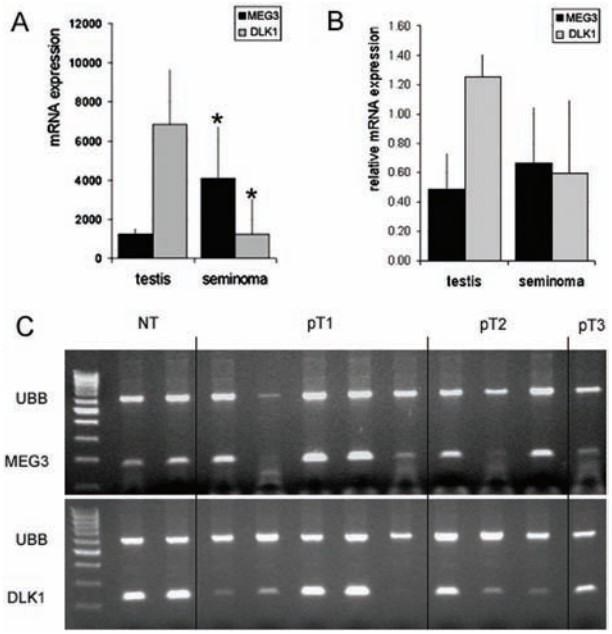

Figure 5. (A) Mean expression level of imprinted genes MEG3 and DLK1 in 3 normal testicular tissues and 40 seminoma specimens as assessed by microarray analyses. Error bars represent the SD amongst the single samples. Asterisks indicate a significant regulation compared to normal testicular tissue. (B) The results of three independent semiquantitative RT-PCR experiments for MEG3 and DLK1 expression in two normal and nine tumour tissues. (C) Semiquantitative RT-PCR for MEG3 and DLK1 mRNA expression in normal and tumour tissues as densitometric analysed in B.

expressed in nine seminoma specimens with a median of 34 % compared to normal testicular tissue.

Interestingly, two genes associated with spermatogenesis were significantly upregulated in progressing tumour stages, as is shown in table 3: the gene BRD2 (D42040, S78771) encoding the bromodomain-containing 2 protein naturally expressed in diplotene spermatocytes, round spermatids and at low levels in spermatogonia [29] and NASP (NM_002482, AW003362), which encodes the nuclear autoantigenic sperm protein found in spermatocytes, spermatids and spermatozoa [30].

Discussion

In recent years, many genes have been described to be associated with the pathogenesis of carcinoma in situ of testicular germ cell tumours [31] and seminoma in the early tumour stage pT1 [32]. Here, we present for the first time a global expression profiling of 40 seminoma specimens of different tumour stages including samples from tumour specimens invading the epididymidis and the spermatic cord (pT3). Beside genes already described as associated with seminoma, we demonstrate here a tumour stage, dependent increase in additional genes such as the transcription factors ETV4 and TEAD4.

Hierarchical cluster analyses distinguished seminoma from normal testicular tissue, but revealed no differences between the tumour stages using a global profiling of a large set of genes. However, hierarchical cluster analysis separated seminoma specimens into three groups depending on the expression of genes participating in the inflammatory response. An impact of inflammation on tumour progression seems likely but needs further investigations.

The transcription factor ETV4 was one of the most upregulated genes during progression of seminoma. Studying the literature, we found ETV4 in a gene array list with transcripts associated with carcinoma in situ [31], indicating an activation of ETV4 already in the early step of tumourigenesis. We found a continuous upregulation from tumour stage pT1 to pT3, indicating an active role of ETV4 in tumour progression. ETV4 has been described as transactivating multiple MMP genes such as MMP1 (collagenase 1), MMP3 (stromelysin 1), MMP7 (matrilysin) and MMP9 (gelatinase B), and plays an important role in tumour invasion, as shown for breast and gastric cancer [25, 33, 34]. From our analyses, *ETV4* seems to regulate the transcript levels of MMP2 and ADAM15 in testis. MMP2 encodes gelatinase A, which degrades type IV collagen, the major structural component of basement membranes, and which is known to be extensively developed in testis [35]. MMP2 expression was slightly increased in seminoma samples. However, analyses of ETV4 protein in normal testicular tissue gave a positive staining in spermatogonia. Moreover, MMP2 expression is documented in these testicular areas and it seems to be involved in germ cell movement along the Sertoli cells [36]. As shown in the present study, MMP2 could play, in addition, a role in the pathogenesis of seminoma.

ADAM15 (metargidin) encodes disintegrin and metalloprotease domain 15, which is a member of the ADAM protein family of membrane-anchored proteins structurally related to snake venom disintegrins. Recently, ADAM15 expression has been described for gastric and lung tumour specimens [37, 38]. Nothing is known about regulation of ADAM15. In our analyses, expression of this gene correlated with that of ETV4 indicating a mutual regulation of both genes. Thus, ETV4 represents a candidate gene that may be involved in the tumour progression of seminoma by an activation of MMP2 and ADAM15 leading to degradation of the extracellular matrix and migration of seminoma cells.

Another highly upregulated transcription factor during progression of seminoma was TEAD4 (TEF3, RTEF1), encoding a member of the transcriptional enhancer factor (TEF) family. Mouse TEF-1 members are differentially expressed during embryogenesis [39] and two different microarray studies showed an upregulation of TEAD4 in embryonic stem cells [40, 41]. Recently, profiling of

samples with carcinoma in situ revealed an enhanced expression of TEAD4 in seminoma precursor cells [31]. Here, we demonstrate that TEAD4 is upregulated in progressing tumour stages. TEAD4 has been localised to vascular endothelial cells and is positively associated with hypoxia-dependent VEGF activity in an endothelial cell line [42]. Thus, a continuous upregulation of TEAD4 in seminoma might possibly stimulate the proliferation and angiogenesis in the tumour tissue.

Gene imprinting may have an effect on tumourigenesis [43]. Seminomas show a consistent expression of both parental alleles of the IGF2 and H19 genes probably due to impaired imprinting [44]. Applying microarrays and RT-PCR analyses, we found an independent regulation of DLK1 and MEG3. Similarities between the DLK1/MEG3 domain and that of the IGF2/H19 locus have previously been noted [45]. We associated for the first time the expression of MEG3 with adult testicular tissue and the constant expression of MEG3 in the tumour tissue may reflect a general erased imprinting, as reported for the H19/IGF2 genes in seminoma.

Though all tumour tissues were classified as pure seminoma, at a representative tissue section from the same tissue as used for the RNA extraction, some testis-specific genes were expressed in seminoma. We observed that the FSHR gene typical for Sertoli cells was still present in some seminoma specimens, supporting earlier reports about persistent Sertoli cells in seminoma tissues [46]. On the other hand, we found seminoma specimens expressing markers for Leydig cells such as INSL3 or DLK1 [28], indicating the presence of these cells within seminoma, as discussed elsewhere [47]. Further investigations should elucidate if genes characteristic for Sertoli and Leydig cells derive from these cell populations that represent a second population within the pure seminoma. Two other testis-specific genes were upregulated in seminoma: BRD2, the bromodomain-containing gene [29], and the gene NASP coding for a histone H1-binding protein [48]. The role of these genes for tumourigenesis remains to be further clarified.

Whether the differences between the samples of seminoma tissue are generated by the developing tumour or by tissue displacement due to failure of spermatogenesis or additional leucocytic infiltration remains to explored. Taking all these possibilities into consideration, ETV4 and TEAD4 are probably the most favourable candidates as transcription factors involved in the progression of seminoma, and their role as well as downstream signalling needs further investigations.

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