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Original Article

HMGA2 expression distinguishes between different types of postpubertal testicular germ cell tumour

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Abstract

The group of postpubertal testicular germ cell tumours encompasses lesions with highly diverse differentiation – seminomas, embryonal carcinomas, yolk sac tumours, teratomas and choriocarcinomas. Heterogeneous differentiation is often present within individual tumours and the correct identification of the components is of clinical relevance. *HMGA2* re-expression has been reported in many tumours, including testicular germ cell tumours. This is the first study investigating *HMGA2* expression in a representative group of testicular germ cell tumours with the highly sensitive method of quantitative real-time PCR as well as with immunohistochemistry. The expression of *HMGA2* and *HPRT* was measured using quantitative real-time PCR in 59 postpubertal testicular germ cell tumours. Thirty specimens contained only one type of tumour and 29 were mixed neoplasms. With the exception of choriocarcinomas, at least two pure specimens from each subgroup of testicular germ cell tumour were included. In order to validate the quantitative real-time PCR data and gather information about the localisation of the protein, additional immunohistochemical analysis with an antibody specific for HMGA2 was performed in 23 cases. Expression of *HMGA2* in testicular germ cell tumours depended on the histological differentiation. Seminomas and embryonal carcinomas showed no or very little expression, whereas yolk sac tumours strongly expressed HMGA2 at the transcriptome as well as the protein level. In teratomas, the expression varied and in choriocarcinomas the expression was moderate. In part, these results contradict data from previous studies but HMGA2 seems to represent a novel marker to assist pathological subtyping of testicular germ cell tumours. The results indicate a critical role in yolk sac tumours and some forms of teratoma.

Keywords: testicular germ cell tumour; HMGA2; seminoma; embryonal carcinoma; yolk sac tumour; teratoma; choriocarcinoma; qRT-PCR; immunohistochemistry; biomarker

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Introduction

Testicular germ cell tumours (TGCTs) are relatively rare, but in many countries they represent the most prevalent cancer in men between 15 and 40 years of age [1]. For unknown reasons its incidence has increased significantly over recent decades in many populations globally [1,2]. Nevertheless, the worldwide frequency varies considerably between different races and countries, with several European countries showing the highest incidences [1,3,4]. In these regions, up to one in 200 men is affected [3]. The

assumed originating cells are primordial germ cells, which undergo malignant transformation thus becoming an intratubular germ cell neoplasia undifferentiated (ITGCNU), formerly referred to as testicular intraepithelial neoplasia or carcinoma *in situ* [reviewed in [5,6]]. In almost all cases of ITGCNU, a TGCT with invasive growth eventually develops [reviewed in [7,8]]. TGCTs are divided into pure seminoma (~50–54%) and nonseminoma (ca. 46–50%) subgroups. The latter also contains mixed tumours [9,10]. This subtyping is of clinical and prognostic relevance [10–12]. Nonseminomas display different degrees of differentiation from embryonal carcinoma (EC) to mature teratoma and display embryonic and extraembryonic differentiation [8,13]. Mixed forms of two or more nonseminomas, or seminoma and nonseminoma, are common: between 13 and 54% of tumours have been reported to contain mixed histology [14,15]. Although a panel of immunohistochemical biomarkers helps to differentiate tumour subgroups, diagnosis can still be a challenge [16].

High-mobility group AT-hook (HMGA) proteins are small and highly charged, consisting of three DNA-binding domains and an acidic carboxy-terminal tail. As architectural transcription factors they lack intrinsic transcription factor capacity but interact with nuclear proteins and enhance or silence transcription through changes in chromatin structure [reviewed in [17,18]]. There are four known HMGA proteins in humans (HMGA1a, HMGA1b, HMGA1c and HMGA2), encoded by two genes [reviewed in [19,20]]. High *HMGA* expression has been detected at embryonic and foetal stages in mammalian tissues. Conversely, no or only very low *HMGA2* expression has been identified in adult tissue; slightly higher levels have been reported for *HMGA1* in some tissues [21–25]. The reactivation of *HMGA* expression has been reported for a multitude of tumours [reviewed in [19,26,27]. Chieffi et al. [28] found *HMGA2* to be critically involved in spermatogenesis in mice. Furthermore, Di Agostino *et al*. [29] found that *HMGA2* interacts with Nek2 in a MAPK-dependent manner in mouse spermatogenesis. In addition to the participation in normal testicular processes, *HMGA2* has also been suggested as a marker for testicular cancer [30 and reviewed in [5,31,32]]. Franco *et al*. [30] showed moderate to high expression of *HMGA2* in ECs and yolk sac tumours (YSTs).

The aim of this study was to investigate the role of *HMGA2* in postpubertal germ cell tumours of the testis. For the first time, highly sensitive quantitative real-time PCR (qRT-PCR) has been applied in combination with immunodetection, to allow more distinctive differentiation of expression levels of *HMGA2* in the subgroups. Another key aspect was determining whether protein level could serve as a diagnostic marker for clinical application.

Methods

Tissue samples

Formalin-fixed paraffin-embedded (FFPE) tumour tissue and snap-frozen samples of normal testis were collected at the Department of Pathology, Albertinen Hospital, Hamburg, Germany, the Department of Pathology, Clinical Centre Bremen-Mitte, Bremen, Germany, and the Institute of Pathology, Elbe Clinic Stade-Buxtehude, Germany. Additional FFPE samples were collected under the supervision of the Leibniz Institute for Prevention Research and Epidemiology, Bremen, Germany. Pathological examinations were performed after haematoxylin and eosin staining of the samples for diagnostic purposes. In complex cases, additional immunostaining with antibodies specific for PLAP, OCT4, CD30, CD117, glypican 3, AFP and β-HCG was conducted according to the relevant pathology department's protocol. FFPE tissue samples from 59 postpubertal patients with TGCT and three snap-frozen normal testis tissues were examined in the study. Histology was re-evaluated by three of the authors (B.H., T.L. and K.B.) according to the WHO classification. Histological subgroups were: 12 pure seminomas, three mixed tumours with a predominant component of seminoma, 10 pure ECs, 13 mixed tumours with a predominant component of EC, three mixed tumours with two equally predominant components of EC/teratoma or EC/YST, two YSTs, three mixed tumours with a predominant component of YST, six pure teratomas, seven mixed tumours with a predominant component of teratoma (see also Table 1). All samples investigated were initially taken for diagnostic purposes and secondarily used for the present study. Samples were deidentified before their use in this study, in line with the rules of the Helsinki declaration. The study was approved by the local ethics committee (Ärztekammer Bremen, reference number 371).

RNA isolation

Depending on the size of the embedded tissue, FFPE blocks were cut into six to eight sections of 5 μm for each sample using a microtome. Total RNA isolations were performed using the innuPREP Micro RNA Kit (Analytik Jena AG, Jena, Germany) for

Table 1. Overview of all testicular cancer samples

Case	RQ	Sample composition (values relative to cancerous content)						Normal tissue, absolute value (%)	Immunohistochemistry	Patient's age	Tumour size (cm)
		Seminoma (%)	EC (%)	YST (%)	Teratoma (%)	CC (%)	Undetermined (%)				
HT01	7,998	0	4	4	92	0	0	75	–	32	2,8
HT02	3,617	100	0	0	0	0	0	11	–	46	6,5
HT03	9,182	0	50	10	40	0	0	70	–	18	2,7
HT04	0.763	100	0	0	0	0	0	70	–	60	1,6
HT05	0.507	100	0	0	0	0	0	90	–	35	1,3
HT06	0.901	0	100	0	0	0	0	80	–	21	2.6
HT07	3.689	0	100	0	0	0	0	70	+	29	1.9
HT08	0.312	100	0	0	0	0	0	15	–	31	4.5
HT09	130.584	0	40	40	0	0	20	25	+	39	3.5
HT10	46.510	0	0	0	70	30	0	50	+	23	6
HT11	0.865	100	0	0	0	0	0	30	–	55	1.5
HT12V	1.808	0	0	0	100	0	0	20	–	23	1.8
HT13	0.234	100	0	0	0	0	0	80	–	39	1.5
HT14	0.438	60	40	0	0	0	0	30	–	18	2
HT15	1.624	0	100	0	0	0	0	85	–	27	2
HT16	0.733	100	0	0	0	0	0	20	–	41	5.5
HT17	111.724	0	40	40	0	20	0	17	+	28	2.5
HT19	0.1*	100	0	0	0	0	0	70	+	38	2.8
HT20	1.765	100	0	0	0	0	0	90	+	50	1.5
HT22	6.922	0	100	0	0	0	0	64	+	53	1.5
HT23	1.880	0	100	0	0	0	0	90	–	35	3.5
HT24	10.833	0	4	0	96	0	0	75	–	28	0.9
HT25	1.041	75	20	5	0	0	0	56	+	20	2.8
HT26	0.681	100	0	0	0	0	0	10	–	26	3.3
HT27	0.143	100	0	0	0	0	0	30	+	39	6.5
HT28	0.322	100	0	0	0	0	0	85	–	46	2.4
HT29	0.873	0	0	0	100	0	0	15	+	21	2
HT30	74.481	0	0	20	80	0	0	38	+	66	4
HT31	17.595	0	90	5	0	5	0	43	–	37	6.5
HT32	31.621	0	60	30	0	5	5	40	–	24	8
HT33	32.310	0	80	10	0	10	0	25	–	23	4
HT34	6.474	0	96	4	0	0	0	33	–	33	1.8
HT35	0.379	0	0	75	25	0	0	38	+	35	4
HT36	50.418	0	0	100	0	0	0	26	–	36	1.5
HT37	61.414	0	80	20	0	0	0	47	–	22	3.4
HT38	1.594	0	0	5	95	0	0	20	–	35	2.6
HT39	7.565	0	90	10	0	0	0	50	–	47	3
HT40	1.403	0	100	0	0	0	0	11	+	30	n.a.
HT41	14.691	0	59	5	35	1	0	11	–	40	1.2
HT42	109.424	0	0	100	0	0	0	25	+	38	6
HT43	99.796	0	5	10	85	0	0	15	+	31	3.5
HT44	147.842	0	40	18	40	2	0	30	+	24	3.2
HT45	6.866	0	0	0	100	0	0	70	+	43	5.5
HT46	18.707	20	60	20	0	0	0	40	–	19	2.7
HT47	15.294	0	95	5	0	0	0	50	–	48	2
HT48	2.960	0	100	0	0	0	0	80	–	23	1
HT49	0.897	0	0	0	100	0	0	50	–	24	1.8
HT50	0.853	0	0	0	100	0	0	80	–	21	1.5
HT51	626.427	0	10	20	70	0	0	20	+	40	4.5
HT52	230.972	75	5	20	0	0	0	30	+	19	3
HT53	28.455	0	90	10	0	0	0	30	+	18	n.a.
HT54	130.314	0	0	95	5	0	0	41	–	43	3
HT55	13.557	0	95	5	0	0	0	22	+	23	3
HT56	6.136	0	100	0	0	0	0	29	–	29	2.1
HT57	2.301	0	0	0	100	0	0	20	–	n.a.	3.5
HT58	3.549	0	100	0	0	0	0	33	–	43	2
HT59	6.824	0	100	0	0	0	0	38	–	30	4.5
HT62	3.733	0	81	10	9	0	0	70	+	27	n.a.
HT63	119.312	0	50	50	0	0	0	26	+	38	2.5

RQ, relative quantification; *HMGA2* expression, EC, embryonal carcinoma; YST, yolk sac tumour; CC, choriocarcinoma; Immunhistochemistry, sample was used in HMGA2-specific immunhistochemistry investigation; *: set value, expression below detection level (see text for further explanation), n.a., not available.

RNA isolation according to the manufacturer's instructions, with the following modifications: Lysis of the paraffin sections preceding RNA isolation was conducted using TLS-Lysis Solution and Proteinase K from the innuPREP DNA Micro Kit (Analytik Jena AG, Jena, Germany) without prior deparaffinisation. Sections were incubated for 1 h at 60°C and 15 min at 80°C.

cDNA-synthesis and quantitative real-time RT-PCR

RNAs were reverse-transcribed into cDNA by M-MLV Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Real-time PCR was performed using the Applied Biosystems 7300 sequence detection system, software 1.2.3, according to the Taq-Man Gene Expression Assay Protocol (Applied Biosystems, Darmstadt, Germany) in 96-well microtitre plates with a total volume of 20 μl. For the TaqMan gene expression assay for *HMGA2* (assay number Hs00171569, Applied Biosystems, Foster City, USA), each reaction consisted of 2 μl of cDNA reverse transcribed from 25 ng of total RNA, 10 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μl of TaqMan assay and 7 μl of ddH_2O. For the *HPRT* assay, using HPRT FP and HPRT RP primers [33], each reaction consisted of 2 μl of cDNA reverse transcribed from 25 ng of total RNA, 10 μl of TaqMan Universal PCR Master Mix, 600 nM (1.2 μl) of forward and reverse primers, 200 nM (0.2 μl) of probe [33] and 5.4 μl of ddH_2O. Thermal cycling conditions were 2 min at 50°C followed by 10 min at 95°C, 50 cycles at 95°C for 15 s and 60°C for 1 min. In each run, a negative control of previous cDNA synthesis (missing reverse transcriptase) was included for each sample and a nontemplate control of amplification and a nontemplate control of previous cDNA synthesis were included for each plate.

All testing reactions were performed in triplicate. Considering the expression range of *HMGA2*, *HPRT* was chosen as the endogenous control as generally suggested by de Kok *et al*. [34], and as used for testicular samples by McIntyre *et al*. [35], Looijenga *et al*. [36] and Wermann *et al*. [37]. The C_T values of both genes were in concordance (*HMGA2*: 21,914–36,006; *HPRT*: 23,421–37,459). As recommended for FFPE samples [38] the fragment sizes amplified by both assays were small, ranging between 65 and 80 bp; validation of these values was performed via gel electrophoresis of the PCR amplicons (data not shown). Relative quantity (RQ) was calculated using the ddCT method [39]. Snap frozen tissue of normal testis was tested against FFPE from the same sample giving highly comparable results. Because of disposability of snap frozen normal testis tissue, the average of three such tissues was used as calibrator.

Immunohistochemical analysis

Slides utilized for the immunohistochemical analysis were produced using cuts directly adjacent to those used for the qRT-PCR investigation. Immunohistochemical staining for HMGA2 (rabbit polyclonal anti-HMGA2-P3, Biocheck, Inc., Forster City, USA) was performed using a detection kit (DAKO ChemMate; DAKO, Glostrup, Denmark) and a semiautomated stainer (DAKO; TechMate) according to the specifications of the manufacturer. For antigen retrieval, the slides were treated in a PT Link module (DAKO) using the EnVision^{TM} FLEX Target Retrieval Solution, low pH (DAKO). The antibody dilution used was 1:1000. Term placenta was used as a positive control whereas negative control was performed by omission of the primary antibody.

Interpretation of HMGA2 staining was done using a Zeiss Axioplan (Carl Zeiss Microscopy GmbH, Göttingen, Germany) microscope. Immunoreactivity in the nucleus was considered positive (although perinuclear granulation in cytoplasm was observed occasionally). In each slide, three to five high-power fields were rated. Staining extent was scored by multiplying intensity of staining (0: no staining, 0.5: very weak, 1: weak, 1.5: weak-moderate, 2: moderate, 2.5: moderate-strong and 3: strong) by percentage of stained tumour cells. Lack of available tissue was one of the reasons why we did not perform IHC in all cases analysed by qRT-PCR.

Statistical analysis

RQ values and immunohistochemical scores were described by number of values, arithmetic means, standard deviations and minimum and maximum. Boxplots were used to summarize the distribution of data values. Averages of immunohistochemical scores were compared by the Wilcoxon two-sample rank sum test. The ability of the RQ value to discriminate between tumour subgroups was quantified for all pairs of tumour subgroups by sensitivity and specificity, obtained from Receiver-Operator-Characteristics (ROC) analysis, thereby using a normal approximation of the empirical data. This analysis was performed for all samples containing only a single type of tumour. The relationship between lg(RQ) and the proportion of tumour components – these expressed as proportion of the total section area – was analysed by linear regression. An intercept was omitted from the

Table 2. *HMGA2* expression in pure tumours

Case	RQ	Type of tumour
HT02	3.617	seminoma
HT04	0.763	seminoma
HT05	0.507	seminoma
HT08	0.312	seminoma
HT11	0.865	seminoma
HT13	0.234	seminoma
HT16	0.733	seminoma
HT19	0.1*	seminoma
HT20	1.765	seminoma
HT26	0.681	seminoma
HT27	0.143	seminoma
HT28	0.322	seminoma
HT06	0.901	EC
HT07	3.689	EC
HT15	1.624	EC
HT22	6.922	EC
HT23	1.880	EC
HT40	1.403	EC
HT48	2.960	EC
HT56	6.136	EC
HT58	3.549	EC
HT59	6.824	EC
HT36	50.418	YST
HT42	109.424	YST
HT12V	1.808	teratoma
HT29	0.873	teratoma
HT45	6.866	teratoma
HT49	0.897	teratoma
HT50	0.853	teratoma
HT57	2.301	teratoma

RQ, relative quantification; EC, embryonal carcinoma; YST, yolk sac tumour; *, set value; expression below detection level (see text for further explanation).

regression equation, because a tumour proportion of zero is by definition associated with $\lg(RQ) = 0$. To allow for the logarithmic transformation of all values, the RQ of zero observed in one case was replaced by $RQ = 0.1$. This value still lies below the smallest observed RQ value. All cases were included in this analysis. A p value of less than 0.05 was considered significant, a p value of less than 0.001 highly significant. Statistical analyses were undertaken using the SAS/STAT and SAS/GRAPH software (version 9.2 for Windows, copyright 2002–2008 SAS Institute Inc.), and the R software [40].

Results

qRT-PCR analysis

Fifty-nine FFPE samples of human TGCTs were tested for the expression of *HMGA2* (Table 1). Of these tumours, 30 were pure tumours (12 seminomas, 10 ECs, two YSTs and six teratomas), 29 were mixed GCTs. These were accompanied by three snap-frozen normal testicular samples used for normalisation. Overall, the samples showed an *HMGA2* expression level between 0.143 and 626.427: this relates to a range of about 1–4381. For one sample, the expression was 0, ie the expression was below the detection limit.

Table 3. *HMGA2* expression in pure tumours by group

Type of tumour	*n*	Average	St dev
Seminoma	12	0.904	1.004
EC	10	3.589	2.292
YST	2	79.921	41.724
Teratoma	6	2.266	2.332

n, number of cases; St dev, standard deviation; EC, embryonal carcinoma; YST, yolk sac tumour.

Focussing on the samples with only one tumour subgroup, there was a clear classification between the groups (Tables 2 and 3, Figure 1). Seminomas showed the lowest values; with two exceptions, all measured data were below the expression in normal tissue. ECs and teratomas showed slightly elevated levels, while the levels expressed by YSTs were by far the highest. This clustering could be visualized by aligning the samples by level of expression (Figure 2, including the mixed tumours).

To statistically validate the visual impression on discriminatory ability, ROC analyses were performed (Table 4). Comparisons involving YST showed a sensitivity of at least 0.988 and a specificity of 0.997, indicating a clear distinction from the other tumour subgroups. These numbers, however, must be treated with caution, since the YST group consisted of only two samples. In addition to the comparison of individual groups, seminomas were tested against all other subgroups. This analysis indicated that seminomas and nonseminomas were separated moderately well by real-time *HMGA2* expression data alone with a sensitivity of 0.912 and a specificity of 0.680.

To analyse qRT-PCR data from samples with two or more tumour components and to accommodate for varying percentages of normal tissue content, a linear regression was calculated between the logarithmic RQ and the tumour components (Table 5, Figure 3). Comparison of observed and predicted RQ values showed good agreement; in particular, no indication of systematic deviation was identified. The model achieved an adjusted coefficient of determination of 0.6625 with a p value of $1.112*10^{-12}$, indicating *HMGA2* expression is dependent on the tumour subgroup. With the exception of seminomas, each subgroup's contribution turned out to be significant or highly significant (see also Table 5). There are four values with large differences between observed and

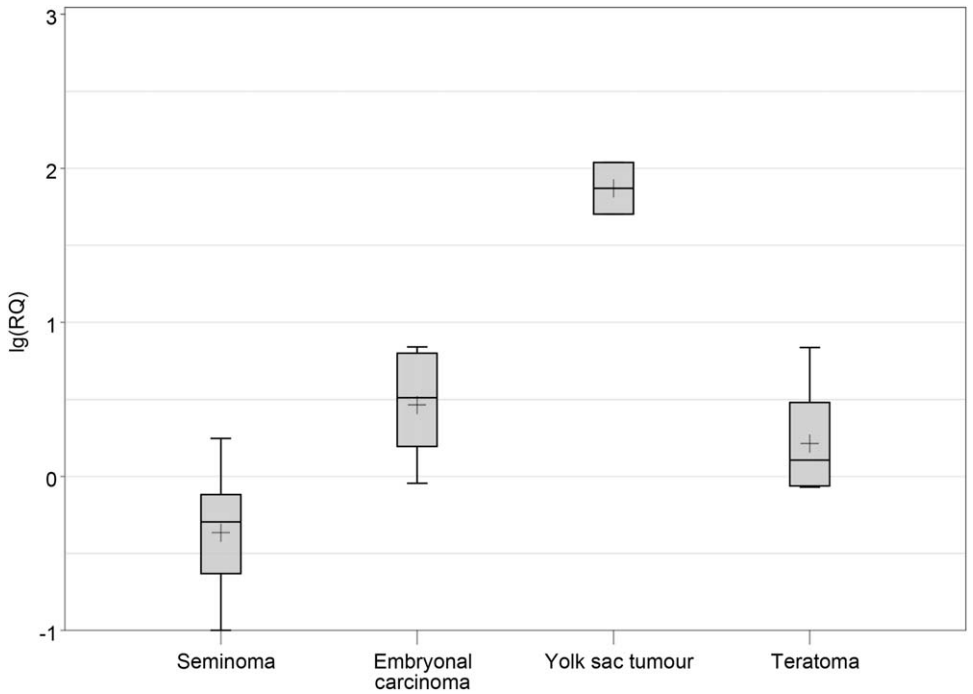

Figure 1. *HMGA2* expression in pure tumours. Boxplots for the relative quantification of *HMGA2* expression in TGCTs. Tumour type at *x*-axis, logarithmic RQ at *y*-axis. Boxes contain the central 50% of all values and a bar at the position of the median, whiskers extend to the extreme values or to 1.5 * box height, whichever is smaller. The plus sign shows the arithmetic mean.

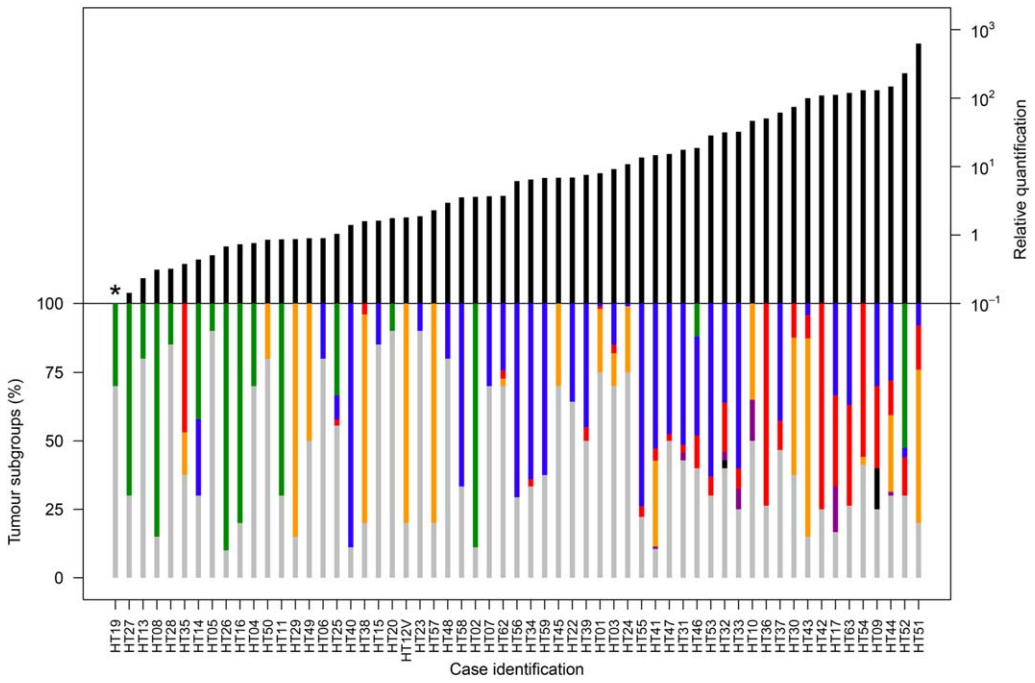

Figure 2. Overview of all testicular cancer samples. Bar plot with RQ in the upper part, tumour composition in the lower part. Samples are arranged by level of *HMGA2* expression. *: set value, expression below detection level (see text for further explanation). Colour code for the tumour composition: green: seminoma, blue: embryonal carcinoma, red: yolk sac tumour, orange: teratoma, purple: choriocarcinoma, black: undetermined, grey: normal tissue.

Table 4. ROC analysis of pure tumours

Type of tumour	*n*		Seminoma	EC	YST	Teratoma
Seminoma	12	sens.		0.868	0.998	0.581
		spec.		0.815	0.999	0.828
EC	10	sens.			0.988	0.883
		spec.			0.997	0.371
YST	2	sens.				0.996
		spec.				0.998
Teratoma	6	sens.				
		spec.				

n, number of cases; sens., sensitivity; spec., specificity; EC, embryonal carcinoma; YST, yolk sac tumour.

predicted expression. HT35 is a clear outlier, with measured expression far below the expected value. As the sample was composed of YST and teratoma, taking the overall results into consideration, much higher expression of *HMGA2* had been expected. HT51 and HT52 showed values higher than expected considering their composition. HT19 is the sample with the set value. In all cases, qRT-PCR data were confirmed by immunohistochemical analysis (see below).

Immunhistological analysis

In 23 cases, the section of the FFPE block following those used for qRT-PCR analysis was investigated for HMGA2 protein expression patterns. Since 14 samples contained two or more histologically different areas, 45 immunohistochemical scores were gathered (Figure 4). Concordant with results from qRT-PCR, the HMGA2 scores in seminomas were nearly zero (mean: 0.0375). One seminoma (HT20) showed focally strong immunoreactivity; all the others were negative. No or very weak staining was observed in EC components, whereas a wide range was observed in teratoma components. There was a tendency for immature structures to be positive, whereas mature teratomas were negative. Exceptions were observed, eg mature glandular structures as goblet cells were often, but not always, strongly positive. On the other hand, primitive neuroepithelium showed weak staining; muscular structures were negative. YST components were strongly positive. Two exceptions were found: one YST was negative (HT25: 5% YST, 75% seminoma, 20% EC) and one showed weak to moderate staining (HT35: 75% YST, 25% teratoma). Both also showed unexpectedly low HMGA2 expression by qRT-PCR. HT51 and HT52, both displaying very high qRT-PCR values, showed equally strong immunostaining. In choriocarcinomas (CCs) syncytiotrophoblasts as well as cytotrophoblasts showed weak to moderate staining (Figure 5, Supplemental Figure 1). HMGA2 expression was

Table 5. Linear regression analysis of the relationship between lg(RQ) and the proportion of tumour components

Tumour component	Estimate	Standard error	*t* value	*p* value
Seminoma	−0.001496	0.003134	−0.477	0.63502
EC	0.014816	0.002875	5.153	$3.72*10^{-6}$
YST	0.030189	0.004875	6.192	$8.38*10^{-8}$
Teratoma	0.01078	0.00327	3.297	0.00173
CC	0.06092	0.029689	2.052	0.04504

Estimate, estimate of the regression coefficient; positive values indicate an RQ value increasing with tumour proportion, *t* value, test statistic for the hypothesis 'Coefficient is zero'; *p* value, level of significance; EC, embryonal carcinoma; YST, yolk sac tumour; CC, choriocarcinoma. The model contains no intercept, as a proportion of zero is by definition associated with lg(RQ) = 0; data: all samples.

seen in 80–100% of syncytiotrophoblastic cells and in 60% of cytotrophoblast components. As we did not find syncytiotrophoblastic cells in our seminoma cases we can neither confirm nor exclude HMGA2 expression in this situation. In normal tissue, HMGA2 was detected in the cytoplasm of the spermatogonial cells. Nuclear expression was weak in spermatocytes and strong in spermatids. Spermatozoa were negative for the protein (Supplemental Figure 1).

A Wilcoxon two-sample rank sum test (Table 6) was conducted to evaluate the separation of tumour entities. Due to multiple testing, a corrected $\alpha = 0.005$ was used. Despite this restriction, significant differences were detected when comparing scores from YSTs with those from ECs and teratomas. Testing seminoma scores against nonseminoma scores resulted in a significant difference in protein level ($p = 0.0154$). Performing the same test with YST values against all other scores revealed a highly significant difference ($p = 3.821*10^{-6}$). Table 7 gives the results from both real-time PCR and immunostaining analysis.

Discussion

An investigation using real-time PCR and immunohistology was performed to study the expression of *HMGA2* in all subgroups of TGCT. Overall, in comparison to normal tissue, seminomas showed a marginal decrease and ECs a slight upregulation. In teratomas, the expression level was variable and appeared to depend on cellular differentiation. CCs (syncytiotrophblastic giant cells and to a lesser extent cytotrophoblasts) and especially YSTs showed considerably increased expression. In normal testicular tissue, low *HMGA2* expression was detected by real-time PCR. This is most likely caused by temporarily high expression in cells involved in spermatogenesis.

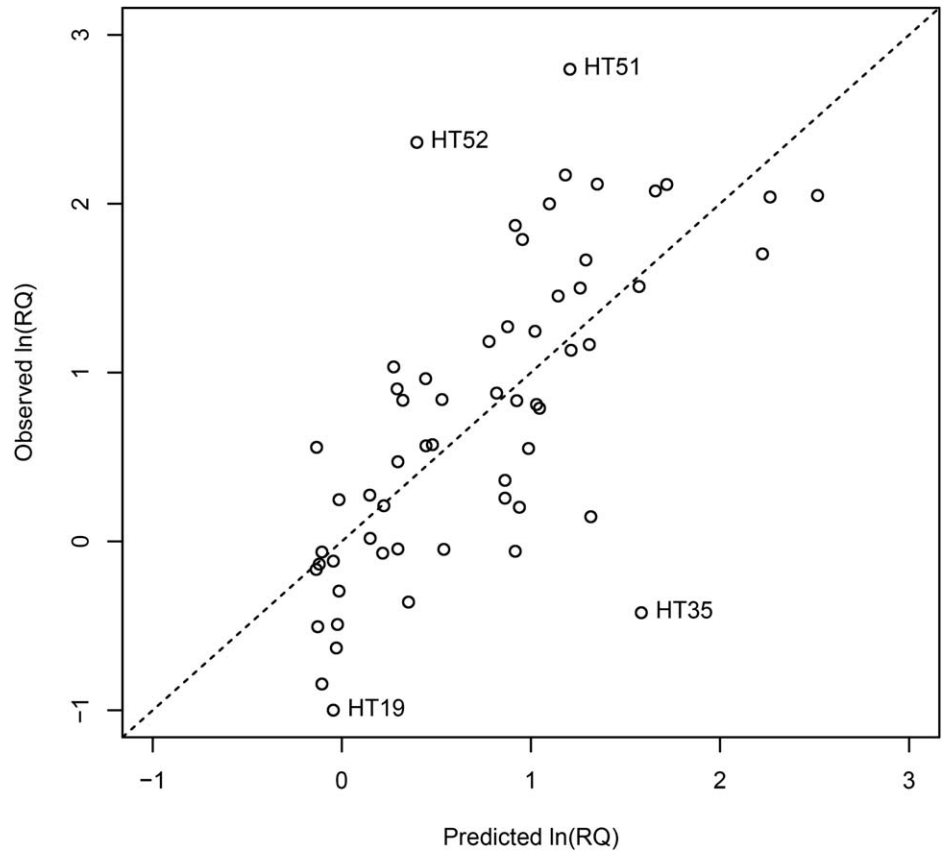

Figure 3. Linear regression analysis of the relationship between lg(RQ) and the proportion of tumour components. Predicted RQ at *x*-axis, observed RQ at *y*-axis, logarithmic scale. Each circle represents one sample. Outliers are marked by case identification (see text for details).

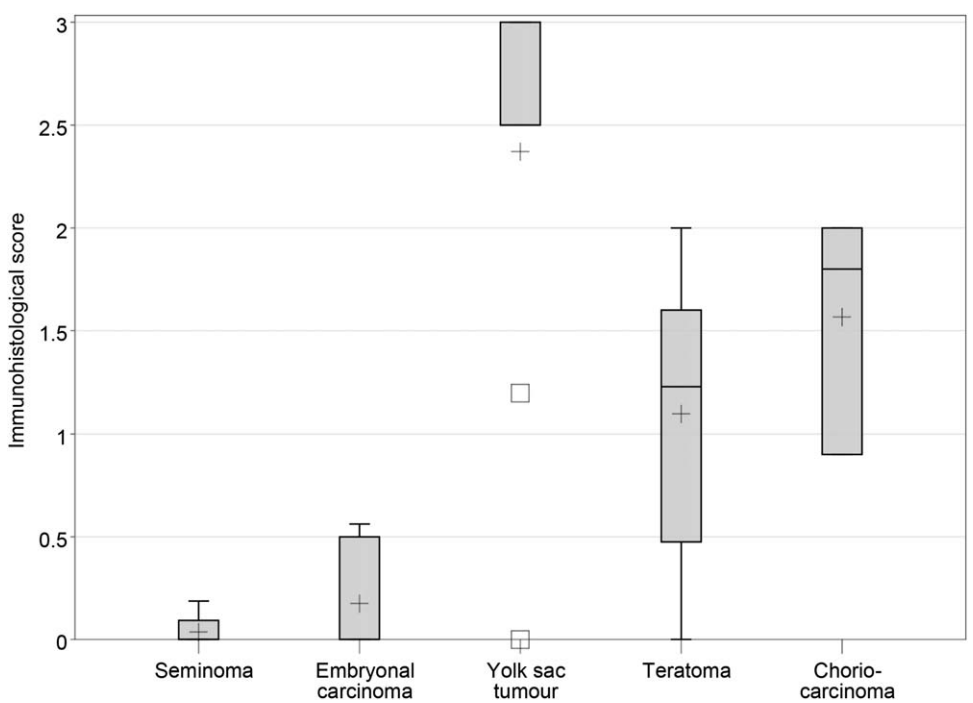

Figure 4. Immunostaining score by type of tumour. Boxes contain the central 50% of all values and a bar at the position of the median, whiskers extend to the extreme values or to 1.5* box height, whichever is smaller. The plus sign shows the arithmetic mean, the rectangles denote outliers.

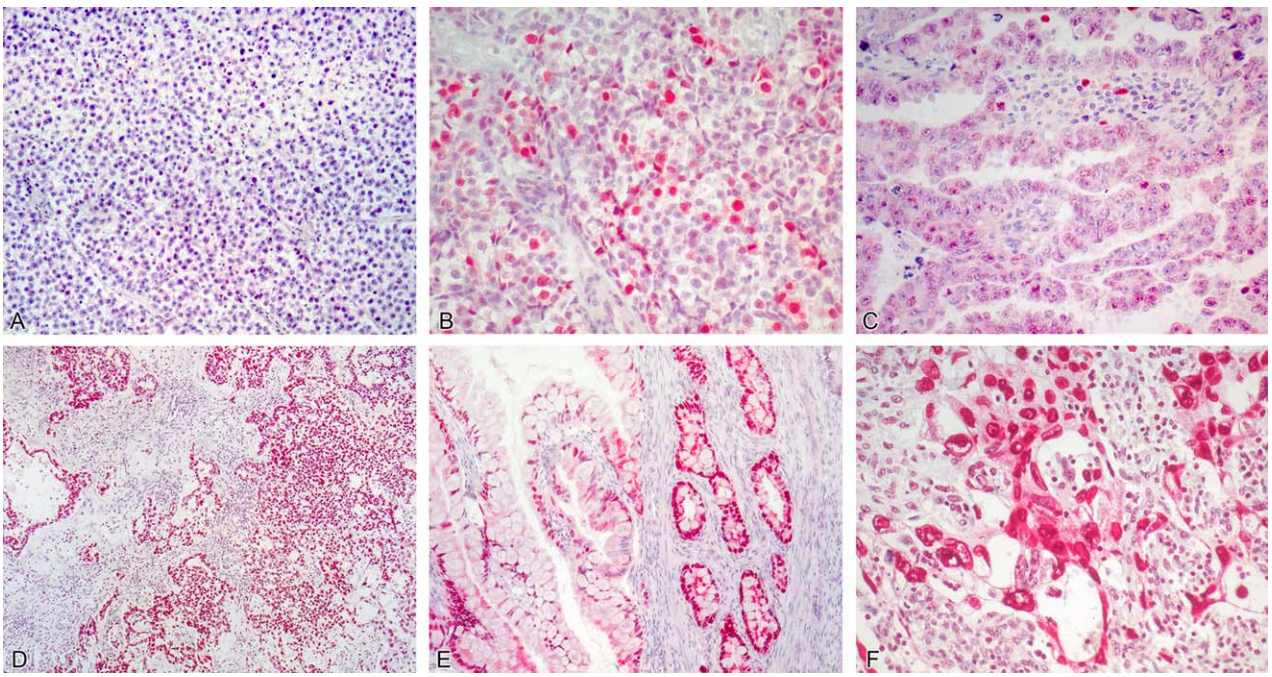

Figure 5. Immunoreactivity for HMGA2 in different tumour subgroups. A: seminoma; B: seminoma with focal HMGA2 reactivity; C: EC with weak granulation; D: YST, microcystic pattern; E: mature teratoma, heterogeneous staining intensity; F: CC intense staining in syncytiotrophoblasts. Original magnifications are given.

Contrary to normal tissue, no such pattern could be detected in most seminomas and immunostaining also showed no HMGA2 expression. Since all pure seminomas contained a percentage of normal tissue, it is plausible that the presence of *HMGA2* mRNA results from that portion. It is also possible that the very low expression detected by real-time PCR is below the threshold of immunohistochemical analysis. One seminoma with relatively high expression (>1) was also investigated using immunohistochemistry. Signals were restricted to one area where single HMGA2 positive cells were scattered in between negative seminoma cells (see Figure 5). One might hypothesize that further transformation of seminoma cells has taken place here. It is known that seminomas can progress into nonseminomas via EC [reviewed in [6]]. As HMGA2 staining in EC was in the range between not detectable and very weak, another type of transformation seems more likely. Nettersheim *et al*. [41] found that the seminoma cell line TCam-2 differentiates into mixed nonseminoma-like cell types without an intermediate step of EC after stimulation with growth-factors TGF-β1, EGF and FGF4. Gopalan *et al*. [42] also suggested a model in which teratoma, YST and CC develop directly from seminoma. Honecker *et al*. [43] found formations of germ cells developing in nonseminomas, but explicitly not in seminomas. This interesting finding remains to be investigated further. At this stage it can just be stated that we detected cells with enhanced self-renewing capacity within one seminoma.

Table 6. Wilcoxon two-sample test comparing the immunohistochemical score by group

Type of tumour	*n*	Seminoma	EC	YST	Teratoma	CC
Seminoma	5		0.3219	0.0065	0.0223	0.0616
EC	14			0.0003	0.0054	0.0147
YST	14				0.0045	0.0544
Teratoma	9					0.3294
CC	3					

n, number of values, EC, embryonal carcinoma; YST, yolk sac tumour; CC, choriocarcinoma. In each cell, the *p* value is stated (2-sided *t* approximation with continuity correction); α with Bonferroni correction: 0.005.

Table 7. Summary of qRT-PCR and immunohistochemical results

Tumour component	*n* (qRT − PCR)	*n* (immunohisto)	*HMGA2* expression
Seminoma	12	5	0
EC	10	14	0–+
YST	2	14	+++
Teratoma	6	9	0–++
CC	–	3	++*

n (qRT − PCR), number of values from real-time PCR; *n* (immunohisto), number of immunohistochemical scores; 0, no expression; (+), very weak expression; ++, moderate expression; +++, strong expression; *, preliminary deduction. Results obtained by linear regression analysis (see Table 5) were used in addition to qRT-PCR data and immunohistochemical scores for this summary.

ECs showed some variation, but always at a low to very low level. Tumours with YST components had a strong tendency towards high *HMGA2* expression, which was clearly confirmed by the immunohistochemical analysis. Teratomas showed a heterogeneous pattern of expression. It seemed that positivity depended on the type of teratoma structure. CC is the most uncommon type of TGCT [10]. Due to the limited availability of CCs, no qRT-PCR-data from homogeneously differentiated tumours could be gathered, and results from the immunohistochemical analysis were limited. Even though the samples present in this study showed a clear tendency, no definitive statement can be made for this subgroup at this point. These data partly confirm the results presented in Franco *et al*. [30]. Besides immunohistology, the authors used western blot analysis and RT-PCR. They also detected high expression of *HMGA2* in YSTs. For seminomas, no expression was reported, which coincides with our results from the immunohistochemical analysis. The residual presence of *HMGA2* expression is likely due to either the high sensitivity of this method, or to the presence of normal cells. In three of six pure teratomas in our study, qRT-PCR values were below one, the other three were slightly elevated. Taking the immunohistochemical analysis into account, and including mixed tumours with a teratoma component, teratomas turned out to be mostly positive, with only one exception of a pure mature teratoma without any positive staining. This is in contrast to the findings presented by Franco *et al*. [30] who did not find expression of *HMGA2* in teratomas. As they did not discriminate between immature and mature teratoma components, direct comparison cannot be performed. Even though it seems unlikely, it is not impossible that all of the 15 samples investigated by Franco *et al*. [30] were mature forms. Results for EC could not be reproduced: Franco *et al*. [30] reported one EC with moderate expression while 14 others showed high expression. This is in clear contrast to our results, for which we can not offer a straightforward explanation. Murray *et al*. [44] also reported positive results for EC. The authors investigated the LIN28/let-7 pathway in malignant germ cell tumours, and found a strongly negative correlation between LIN28 and let-7. As a minor aspect, the study also encompassed the analysis of the expression of *HMGA2*, but, due to the limited sample size of ECs ($n = 3$: one postpubertal, two paediatric), the results might not be representative for this particular group of neoplasms.

As HMGA2 is a nuclear protein, expression analysis generally focuses on nuclear staining [45–49]. Nevertheless, in the present study cytoplasmic positivity was observed occasionally in teratomas and to a lesser extent also in EC (Supplemental Figure 1). Other researchers have made similar observations in different tissues [47,50,51]. Taking into account these data and using a highly specific antibody, it seems less plausible that the cytoplasmic signal was artefactual. The task of developing an approach to clarify these findings remains.

Bearing a high mortality until the mid-1970s, today patients with a TGCT have a 5-year survival rate of 90–95% [52–54]. The remaining deaths are mostly due to chemoresistance of certain subgroups of TGCT: teratomas are benign-appearing, but metastases can form in 29% [15]. Mature teratomas have lost their embryonic features and are therefore completely resistant to cisplatin-based chemotherapy and other clinical treatment strategies [55]. After initial chemotherapy in patients with mixed TGCT with a portion of teratoma, teratoma can be found in the residual mass in 82% of cases [56]. CC metastasises early, therefore a high percentage of mixed tumours show a poor prognosis [15,57]. For several years, different studies found an amount of >50% EC to confer a higher risk for relapses [58]. Recently a follow-up study showed that the any presence of EC, independently of the amount, increases the relapse risk [59]. This illustrates the importance of determining the composition of the particular tumour. A proven set of antibodies for determination of the subgroups exists. Nevertheless, identification can pose a challenge for the pathologist [16,30], and a false diagnosis rate of 4–32% has been reported [60–62].

HMGA2 expression in YST turned out to be different from other types of TGCTs. To a somewhat lesser degree, immunohistochemical staining was also positive for (immature) teratoma components and CC. YST has a wide variety of growth patterns; it can be difficult to differentiate from seminomas, which is of therapeutic relevance [63]. AFP, the only immunohistochemical marker of YST for a long time, often shows only focal staining [15,64]. In recent years glypican-3, SALL4 and LIN28 have been established as diagnostic markers [15,64–68]. Glypican-3 has a higher sensitivity than AFP, but also shows focal staining [64,69]. This was confirmed in the present study. In most glandular growth patterns glypican-3 and HMGA2 showed identical expression. Yet much more HMGA2 positivity was observed in primitive reticular components with non-cohesive cells (Supplemental Figure 1G, 1I). Therefore HMGA2 staining seems to be more sensitive than glypican-3. HMGA2 shows expression (to a somewhat lesser degree) also in (immature) teratomas and CCs. Ota *et al*. [64] also found glypican-3

positivity in teratoma and CC. The specificity of HMGA2 and glypican-3 is therefore comparable for germ cell tumours. SALL4 and LIN28 are both sensitive markers. SALL4 is positive in all germ cell tumour subgroups including ITGCNU [65]. Therefore, it cannot be used to distinguish between different subgroups. LIN28 is sensitive for ITGCNU, seminomas, ECs and YSTs [66]. β-HCG is an established marker for CC, but as Lempiäinen *et al*. [70] showed recently, it can also be positive in ECs. Furthermore the authors found no expression in one of three pure CCs and two mixed TGCTs containing a CC component.

Our data suggest that the use of a HMGA2-specific antibody could be a sensible addition to existing markers and potentially help to decrease the rate of false diagnoses. A study composed of a larger number of FFPE and fresh-frozen samples, including a representative number of CCs, could bring this method even closer to clinical application. In addition, investigation of the expression of *HMGA2* in ovarian and extragonadal germ cell tumours would be of particular interest.

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Authors' contributions

L.K. and J.B. conceived study. L.K., A.G. and J.B. designed study. L.K. carried out molecular genetic studies. A.G., L.K., B.H., T.L. and K.B. carried out immunohistological studies. B.H., T.L. and K.B. performed pathological analysis. B.H., T.L., K.B., G.B. and K.G. provided study material. W.W., L.K. and A.G. carried out statistical analysis. L.K. and A.G. wrote the manuscript. All authors revised the manuscript and had final approval of the submitted and published versions.

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SUPPLEMENTARY MATERIAL ON THE INTERNET

Additional Supporting Information may be found in the online version of this article.

Supplemental Figure 1. Examples of HMGA2 immunoreactivity in normal testis tissue and different tumour subgroups. A: normal seminiferous tubules showing nuclear staining in spermatocytes and spermatids and weak cytoplasmic staining in spermatogonial cells, B: EC surrounded by YST, C: mature teratoma, glandular structure, D: immature teratoma, mesenchymal appearance, E: same as D, negative glypican-3 staining shows that no YST components are present, F: mature teratoma, glandular structures positive, muscular structures negative, G: glypican-3 staining in YST components restricted to glandular growth patterns, H: same as G, HMGA2 staining in YST is also strongly positive in primitive reticular components with noncohesive cells. Original magnifications are given.

