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Research paper

Gene expression and immunohistochemical analyses identify SOX2 as major risk factor for overall survival and relapse in Ewing sarcoma patients

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ABSTRACT

Background: Up to 30–40% of Ewing sarcoma (EwS) patients with non-metastatic disease develop local or metastatic relapse within a time span of 2–10 years. This is in part caused by the absence of prognostic biomarkers that can identify high-risk patients and thus assign them to risk-adapted monitoring and treatment regimens. Since cancer stemness has been associated with tumour relapse and poor patient outcomes, we investigated in the current study the prognostic potential of SOX2 (sex determining region Y box 2) – a major transcription factor involved in development and stemness – which was previously described to contribute to the undifferentiated phenotype of EwS.
Methods: Two independent patient cohorts, one consisting of 189 retrospectively collected EwS tumours with corresponding mRNA expression data (test-cohort) and the other consisting of 141 prospectively collected formalin-fixed and paraffin-embedded resected tumours (validation-cohort), were employed to analyse SOX2 expression levels through DNA microarrays or immunohistochemistry, respectively, and to compare them with clinical parameters and patient outcomes. Two methods were employed to test the validity of the results at both the mRNA and protein levels.
Findings: Both cohorts showed that only a subset of EwS patients (16–20%) expressed high SOX2 mRNA or protein levels, which significantly correlated with poor overall survival. Multivariate analyses of our validation-cohort revealed that high SOX2 expression represents a major risk-factor for poor survival (HR = 3·19; 95%CI 1·74–5·84; $p < 0·01$) that is independent from metastasis and other known clinical risk-factors at the time of diagnosis. Univariate analyses demonstrated that SOX2-high expression was correlated with tumour relapse ($p = 0·002$). The median first relapse was at 14·7 months (range: 3·5–180·7).
Interpretation: High SOX2 expression constitutes an independent prognostic biomarker for EwS patients with poor outcomes. This may help to identify patients with localised disease who are at high risk for tumour relapse within the first two years after diagnosis.

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Research in context

Evidence before this study

One third of EwS patients with localised disease experiences relapse during follow-up. So far, no study has succeeded in identifying a broadly available *bona fide* stratification biomarker to predict which patients will develop EwS recurrence. SOX2 was studied in EwS by two different groups and linked to stemness and tumourigenicity in *in vitro* and *in vivo* models. However, the expression of SOX2 in both studies was either not analysed in tumour material derived from EwS patients or was analysed only in small series and not associated with clinicopathological features, such as overall survival (OS), tumour volume, metastasis at diagnosis, relapse, and histological response.

Added value of this study

This is the first study to evaluate the prognostic potential of SOX2 at the mRNA and protein levels in EwS patients. Here, we report, based on the analysis of two independent cohorts (a retrospective mRNA-cohort and a prospective TMA-cohort) consisting of 189 and 141 cases, respectively, that high SOX2 is significantly correlated with poorer patient outcome. In both cohorts, the association of high SOX2 expression with OS was independent from the presence of metastasis at diagnosis. A univariate analysis showed in the TMA-cohort that SOX2-high expression is correlated with tumour relapse ($p = 0.002$), providing, for the first time, a promising and widely available prognostic biomarker to stratify high-risk patients with localised disease.

Implications of all the available evidence

A pivotal role of SOX2 in tumourigenesis has been consolidated in several tumours, including EwS. Although SOX2 does not represent a druggable target, in this study we suggest employing SOX2 as a stratification marker to identify high-risk patients and initiate molecular studies and clinical trials aimed at developing more personalised therapies for these patients. Moreover, patients with SOX2-high should be monitored more closely during the follow-up so that timely intervention can be made in cases of relapse.

1. Introduction

Ewing sarcoma (EwS), the second most common bone and soft tissue cancer in children and adolescents, potentially arises from neuroectodermal or mesodermal mesenchymal stem cells [1]. In fact, EwS tumours display a largely undifferentiated and 'stemness' phenotype, which is believed to contribute to its clinical aggressiveness [1]. Genetically, the hallmark of EwS is chromosomal translocations that generate chimeric proteins through fusion of the *EWSR1* (*Ewing sarcoma breakpoint region 1*) gene to various members of the ETS (E26 transformation specific) family of transcription factors, in 85% *FLI1* (*Friend leukemia virus integration 1*) [2–4]. These EWSR1-ETS fusion oncoproteins act as aberrant transcription factors that promote tumour initiation and progression by massively rewiring the cellular transcriptome and spliceosome [1].

Of EwS patients, 60–75% benefit from multimodal therapy [5,6]. Consequently, >30% of patients show a limited response to treatment, which is often first noted based on assessment of their histological response following neoadjuvant chemotherapy [7]. Since limited treatment response may be associated with early relapse [8], upfront identification of high-risk patients is essential to assigning them to adequate treatment regimens. Despite several clinicopathological features (such as tumour volume, histological response, tumour site and age at diagnosis) have been associated with high-risk disease, the presence of metastasis at diagnosis remains, thus far, the major factor for risk-stratification in EwS patients [9]. However, for patients with localised disease, risk prediction remains difficult, as no *bona fide* prognostic biomarkers independent from metastasis are available [1,10].

A previous report has indicated that stemness in EwS may be mediated *via* EWSR1-FLI-induced expression of *SOX2* (*sex determining region Y box 2*) [11] – a well-known stemness gene overexpressed in many cancers [12]. We therefore explored, in the current study, the expression pattern of SOX2 in two independent EwS cohorts and investigated whether SOX2 may serve as a biomarker for outcome prediction in EwS.

We show that SOX2 is expressed only in ~16–20% of EwS tumours and is associated with very poor patient outcome independent of metastasis at time of diagnosis. Moreover, high expression of SOX2 is significantly correlated with local and/or metastatic tumour relapse. Our findings suggest that detection of high SOX2 expression through immunohistochemistry or RNA-based techniques may constitute a broadly available biomarker for risk-stratification even for patients with localised disease.

2. Materials and methods

2.1. Study populations

For this study, a retrospective test-cohort and a prospective validation-cohort were analysed. Patient characteristics of both cohorts are listed in Table 1.

The study population of the test-cohort consisted of 189 EwS patients whose molecularly confirmed and retrospectively collected primary tumours were profiled at the mRNA level by gene expression microarrays in previous studies [13–16]. To assemble this mRNA-cohort, microarray data generated on Affymetrix HG-U133Plus2.0, Affymetrix HuEx-1.0-st or Amersham/GE Healthcare CodeLink microarrays of the 189 EwS tumours (Gene Expression Omnibus (GEO) accession codes: GSE63157 [13], GSE12102 [14], GSE17618 [15], GSE34620 [16], and unpublished data) provided with clinical annotations were normalised separately as previously described [17]. Based on specific gene expressions, we predicted for each sample the tumour purity via the ESTIMATE algorithm [18], which revealed that all samples had a consensus purity estimation of >60%, corresponding to The Cancer Genome Atlas (TCGA) standard tissue sample requirements (http://cancergenome.nih.gov/cancersselected/biospeccriteria). Only genes that were represented on all microarray platforms were kept for further analysis. Batch effects were removed using the ComBat algorithm [19]. The removal of batch effects was demonstrated by t-SNE analysis of gene expression data before and after the batch correction (appendix fig. 1). This yielded a dataset comprising 189 EwS samples and 13,253 genes. Data processing was done in the statistical language R.

The validation-cohort was composed of 141 EwS patients treated with first-line therapy according to the successive phase III EwS protocols of European Intergroup Cooperative Ewing's Sarcoma Study (EICESS) 92 (1992 to 1998), and EURO-EWING 99 (1999 to 2009) run by the German Society for Paediatric Oncology and Haematology (GPOH). These studies were registered under ClinicalTrials.gov and approved by the appropriate ethics committees. The corresponding patient tumours were prospectively collected and tissue microarrays (TMAs) were constructed as detailed below. This study population included 87 males and 54 females.

2.2. TMA establishment and immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded (FFPE) EwS samples were retrieved from the archives of the Institute of Pathology of the LMU Munich (Germany) and the Gerhard-Domagk-Institute for Pathology of the University of Münster (Germany) with approval from the corresponding institutional review boards. All EwS samples were collected at diagnosis and reviewed by a reference pathologist, and diagnoses were confirmed by the detection of pathognomonic EWSR1-ETS fusion oncogenes by qRT-PCR or the detection of an EWSR1 break-apart by fluorescence-in-situ-hybridisation (FISH). TMAs were constructed as previously described [17]. Each EwS case was represented by at least two cores (each 1 mm in diameter).

For IHC, 4 μm sections were cut, and antigen retrieval was performed using microwave treatment. Slides were incubated with a primary monoclonal rabbit anti-SOX2-antibody (1:100 dilution, D6D9 XP Cell Signaling) for 60 min. Then, the slides were incubated with a secondary anti-rabbit IgG antibody (ImmPress Reagent Kit; peroxidase-conjugated) followed by target detection using AECplus chromogen for 10 min (Dako, K3461). The specificity of the anti-SOX2-antibody used was confirmed in xenografts of the EwS cell line POE in NOD/Scid/gamma (NSG) mice, in which SOX2 expression was silenced by a doxycycline (dox-)inducible shRNA against the 3'UTR of SOX2 (appendix fig. 4B). For quantification of SOX2 immunoreactivity, the average percentage of SOX2-positive nuclei was evaluated by a data-blinded pathologist who examined at least five high-power fields per case. Examples of nuclear SOX2 immunoreactivity are given in Fig. 1D.

2.3. Statistical analyses

In the mRNA-cohort, the optimal cut-off for stratifying patients by SOX2 expression levels was identified by in-house software (GenEx), which tested all possible cut-offs between the 20th and 80th expression percentiles for the largest difference in area under the curves in the Kaplan-Meier analyses. This analysis identified the 80th percentile of SOX2 expression as the optimal cut-off for stratification of the mRNA-cohort in SOX2-high and -low. Statistical analyses were carried out with SPSS 19 (IBM Corporation, Armonk, NY) and SAS 9.2 (SAS Institute, Cary, NC) as described [20]. Event-free survival (EFS) and overall survival (OS) were estimated using the Kaplan-Meier method. OS time was defined as the interval between the date of diagnosis and the date of the patient's final follow-up or death. Group comparisons were calculated using the log-rank test. Multivariate analyses were carried out by applying the Cox proportional hazard method. Differences in proportions between groups were evaluated using chi-square or Fisher's exact tests. The significance level was set at $p < 0.05$ for two-sided testing. No alpha corrections were carried out for multiple testing. Outcome was analysed on an exploratory basis.

2.4. Role of the funding source

The funders of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. Both corresponding authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Table 1
Patients characteristics ($n = 189;141$).

Variable	Test-cohort (mRNA) n (%)	Validation-cohort (TMA) n (%)
Sex		
Male	86 (57·7)	87 (61·7)
Female	63 (42·3)	54 (38·3)
	(missing: 47)	
Age at diagnosis		
<15 years	112 (59·3)	70 (49·7)
≥15 years	77 (40·7)	71 (50·3)
Metastasis at diagnosis		
Absent	53 (69·7)	87 (61·7)
Present	23 (30·3)	54 (38·3)
	(missing: 113)	
Site[a]		
Axial	NA	86 (61·9)
Non-axial	NA	53 (38·1)
		(missing: 2)
Tumour volume		
<200 ml	NA	76 (55·9)
<200 ml	NA	60 (44·1)
		(missing: 5)
Histological response[b]		
Good	NA	67 (79·8)
Poor	NA	17 (20·2)
		(missing: 57)
Status		
Alive	113 (59·8)	84 (59·6)
Dead	76 (40·2)	57 (40·4)
SOX2 expression		
High	38 (20·1)	22 (15·6)
Low	151 (79·9)	119 (84·4)

[a] All extremity localization are non-axial.
[b] NA = not available.

3. Results

3.1. SOX2 is expressed in a subset of EwS patients and is correlated with OS

SOX2 is a well-known transcription factor involved in stemness during normal development and cancer [12]. In 2010, Riggi et al. proposed that EWSR1-FLI1 confers stemness features to EwS cells *via* up-regulation of SOX2 [11]. We therefore reasoned that SOX2 expression levels might be connected to patient outcome.

To test this hypothesis, a test-cohort obtained from a large EwS gene expression dataset comprising 189 samples, for which matched clinical data were available (Table 1), was employed to evaluate mRNA levels of *SOX2*. In contrast to previous suggestions that SOX2 overexpression might constitute a general feature of EwS [11], we found, using a microarray analysis, that *SOX2* mRNA was not or was only minimally expressed in 79·9% (151/189) of tumours. Indeed, only 20·1% (38/189) of the samples exhibited moderate to strong *SOX2* expression levels (Fig. 1A). Stratifying our mRNA-cohort by a cut-off of the 80th

percentile of *SOX2* expression in *SOX2*-low or -high cases, we noted that patients with high intratumoural *SOX2* expression had worse OS than patients with low *SOX2* expression ($p < 0.0001$) (Fig. 1B).

To validate this finding at the protein level, we stained an independent validation TMA-cohort comprising 141 EwS cases with an anti-SOX2-antibody with proven specificity for EwS (appendix fig. 4B). Patient characteristics are reported in Table 1. Scoring of the percentage of nuclear SOX2 positive cells confirmed that the majority of EwS cases (84·4%; 119/141) showed ≤10% positive nuclei (classified as SOX2-low) while only a small subset of patients (15·6%; 22/141) had >10% positive nuclei (classified as SOX2-high) (Fig. 1C,D). Strikingly, applying the cut-off of >10% SOX2 positive nuclei fully confirmed the strong association of SOX2 expression with poor event-free survival (EFS) ($p = 0.0008$) and OS of EwS patients ($p = 0.0004$) (Figs. 1E,F). In both the mRNA-test and TMA-validation cohorts, EwS samples were reviewed by a reference pathologist and diagnosis was confirmed either by the detection of pathognomonic *EWSR1-ETS* fusion oncogenes by qRT-PCR or the detection of an *EWSR1* break-apart by
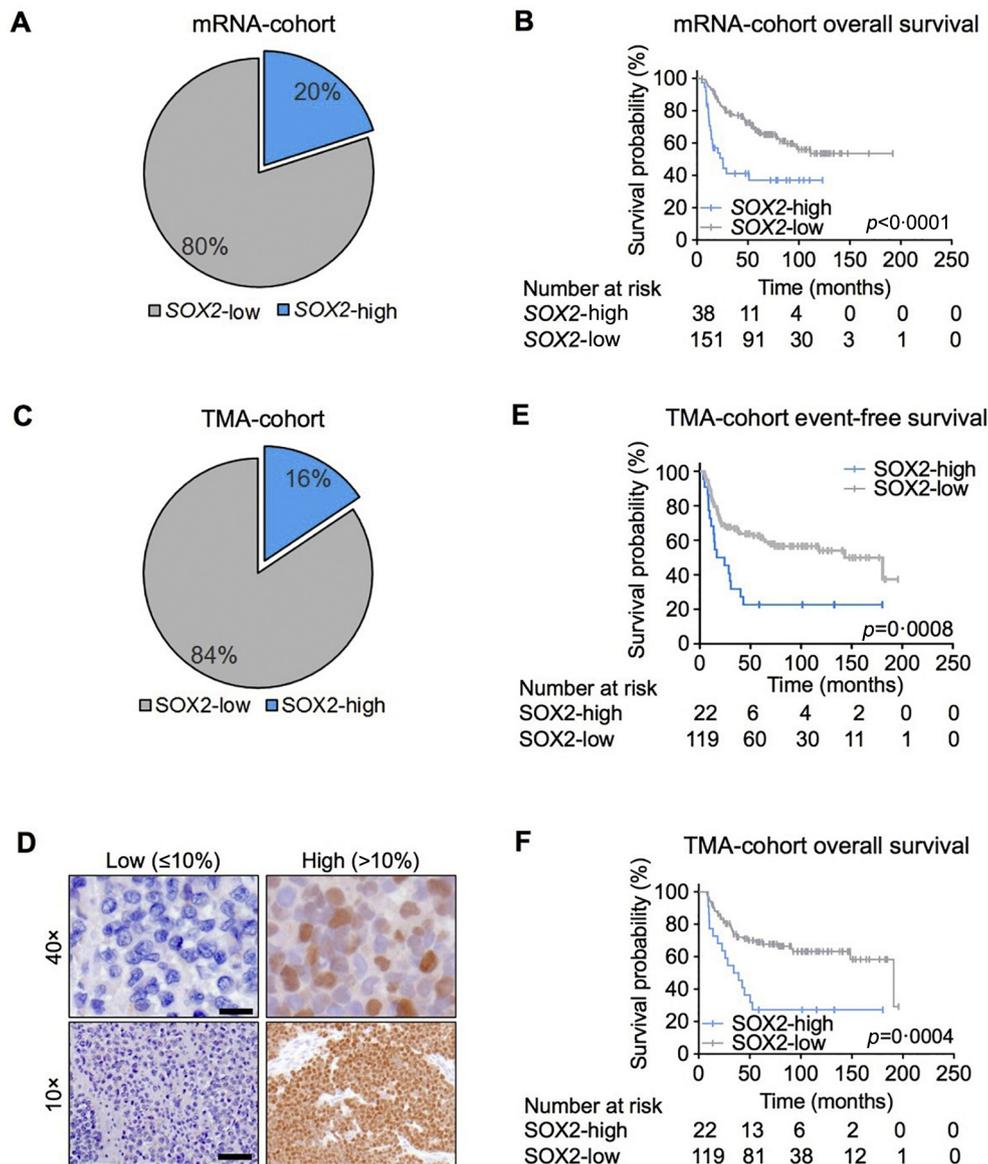

Fig. 1. SOX2 is expressed in a subset of EwS patients and correlates with event-free survival (EFS) and overall survival (OS). (A). Microarray analysis of *SOX2* mRNA expression in 189 primary EwS cases. (B). Kaplan-Meier analysis of OS in 189 EwS patients stratified by their *SOX2* mRNA expression level (cut-off 80th percentile). (C). Analysis of SOX2 protein expression levels in an independent cohort of 141 EwS patients in a TMA by IHC. (D). Representative images for SOX2 IHC (nuclear staining) in EwS patients with different percentages of nuclear SOX2 immunoreactivity. Scale bars = 50 μM (40×) and 200 μM (10×). (E). Kaplan-Meier analysis of EFS in 141 EwS patients stratified by their SOX2 protein expression level (cut-off >10% positive nuclei). (F). Kaplan-Meier analysis of OS in 141 EwS patients stratified by their SOX2 protein expression level (cut-off >10% positive nuclei).

fluorescence-*in-situ*-hybridisation (FISH). To investigate whether the fusion type (*EWSR1-FLI1* type 1 and type 2 and *EWSR1-ERG*) affects *SOX2* expression in EwS, we analysed data obtained from 18 EwS cell lines with different fusion types. These data demonstrated that there is no significant difference in *SOX2* expression between EwS cell lines with different fusion types (appendix fig. 2).

Collectively, these results demonstrated, for the first time, that high SOX2 expression is not a common feature of EwS, but its high mRNA and protein levels may serve as a biomarker for outcome prediction.

3.2. High SOX2 expression is a major risk-factor for tumour associated-death independent from metastasis at time of diagnosis in EwS

To identify factors that may influence patient prognosis, we performed a multivariate analysis in our validation TMA-cohort, as here, additional clinicopathological information beyond OS was available. Interestingly, the multivariate analysis revealed that the major risk-factors were metastatic disease at time of diagnosis at the M2 stage and SOX2-high expression with hazard ratios (HRs) of 4·86 and 3·19 (both $p < 0·01$), respectively. Instead, M0 (HR = R; $p < 0·01$), M1 ($n = 24$; HR = 1·76; $p = 0·13$), age ($≥15$ years; HR = 1·34; $p = 0·28$) and primary axial tumour site (HR = 1·64; $p = 0·11$) did not show a significant impact on survival ($n = 141$; Table 2).

To exclude an interdependency of metastasis at time of diagnosis and SOX2 expression status, we repeated the multivariate analysis considering only patients with localised disease (M0, $n = 87$). In this analysis, SOX2-high expression (HR = 3·22; $p < 0·01$) represented the main risk-factor for survival, followed by age ($≥15$ years; HR = 2·43; $p = 0·04$), whereas primary axial tumour site (HR = 1·66; $P = 0·25$) showed only a tendency to affect survival (Table 3; $n = 87$). In agreement with these findings, in both validation- and test-cohorts, univariate analysis did not show a correlation between SOX2-high and metastasis at time of diagnosis (Table 4, $n = 141$, $p = 0·8$; and Table 5, $n = 76$, p 0·5). Taken together, these findings demonstrate that SOX2-high expression constitutes an independent risk-factor for survival of EwS patients, even in patients with localised disease.

3.3. High SOX2 expression is significantly correlated with tumour relapse

Next, we investigated whether the correlation between SOX2-high expression and OS is associated with histological response, high tumour volume ($≥200$ ml), and relapse, which are risk-factors for worse outcomes [10,21–23].

In our TMA-cohort, data on histological response, tumour volume, and tumour relapse were available for 84, 136 and 141 patients, respectively (Table 4). Our data did not show an association between high SOX2 expression and histological response (Table 4). However, we observed a tendency for an association between SOX2-high and tumour volume. In fact, 58·3% of patients with high SOX2 expression had a tumour volume ≥ 200 ml compared to 41·7% patients with low SOX2 expression ($p = 0·23$) (Table 4). Since the association of SOX2 and higher tumour volume suggested a role for SOX2 in EwS growth, we performed functional *in vitro* and *in vivo* experiments using an established EwS cell line model (POE) for which we generated a derivate with a doxycycline (dox-)inducible shRNA against *SOX2*. The results showed that the knockdown of *SOX2* in POE cells led to strong reduction in cell proliferation, clonogenic growth and anchorage-independent growth compared to cells that expressed a non-targeting control shRNA (appendix fig. 4). Consistently, dox-induced silencing of SOX2 in POE cells xenografted in immunocompromised NSG mice significantly reduced tumour growth (appendix fig. 4).

In addition, we noted a striking correlation between SOX2-high expression and tumour relapse ($p = 0·002$) (Table 4), comprising in 86% of cases metastatic relapse ($n = 39$) or combined metastatic and local relapse ($n = 17$), and in 14% of cases exclusively local relapse ($n = 9$). While only 40·3% of patients with SOX2-low expression had

relapses, recurrence occurred in 77·3% of patients with SOX2-high expression (Table 4), which may explain the poor outcome that was observed for these patients. Indeed, immunohistological assessment of 12 available pairs of primary EwS and relapse samples showed that while SOX2-high expression was only observed in one case at the time of diagnosis four cases exhibited SOX2-high expression at relapse (average percentage of SOX2 expression: 2·75 in primary EwS *vs.* 15·5 in relapse tumours, $p = 0·219$; Wilcoxon matched-pairs signed rank test). In agreement with these observations, gene set enrichment analysis (GSEA) in our mRNA cohort showed that genes co-expressed with *SOX2* in primary EwS tumours are involved in stemness, proliferation, dedifferentiation, and cancer relapse (appendix fig. 5A, appendix table 1).

Collectively, these results suggest that SOX2-high expression may confer worse outcomes for EwS patients by contributing to tumour growth and relapse.

4. Discussion

Up to 30–40% of EwS patients with non-metastatic disease develop local or metastatic relapse within a time span of 2–10 years [24]. Clinical prognostic markers, such as primary dissemination, tumour site, size, age, and histological response to chemotherapy, are established risk-factors used for therapeutic stratification [24]. However, especially for patients with localised disease and otherwise no apparent risk-factors, risk prediction is challenging [25]. Although there is broad consensus that clinical management will benefit from prognostic or predictive biomarkers that can guide therapeutic decisions, there are currently no *bona fide* or broadly available biomarkers that may help predict tumour relapse and outcome among patients with EwS [1,10]. A recent retrospective study on a single cohort of 63 EwS patients with localised disease suggested that detection of a higher tumour-mutational-burden (TMB) in EwS may help to identify patients at risk for relapse among patients with localised disease [26]. However, TMB detection requires sophisticated sequencing technologies that may not be technically applicable in all EwS cases.

In this study, we reported, for the first time, that high mRNA or protein expression of SOX2, which can be determined with routine technologies such as immunohistochemical stains, is a stratification risk-factor for ~16–20% of EwS patients with poor outcomes. Notably, multivariate analyses that included either all or only patients with localised disease have demonstrated that SOX2-high expression represents an

Table 2
Multivariate analysis in all patients (n = 141).

Variable	HR (95%CI)	p
Risk group		
M0 (no met; n = 87)	R[a]	<0·01
M1 (lung met; n = 24)	1·76 (0·85–3·62)	0·13
M2 (other +/− lung met; n = 30)	4·86 (2·65–8·91)	<0·01
SOX2		
High (>10%)	3·19 (1·74–5·84)	<0·01
Age		
≥15 years	1·34 (0·79–2·27)	0·28
Site		
Axial	1·64 (0·90–2·96)	0·11

[a] R = Reference.

Table 3
Multivariate analysis in patients with localised disease (n = 87).

Variable	HR (95%CI)	p
SOX2		
High (>10%)	3·22 (1·36–7·65)	<0·01
Age		
≥15 years	2·43 (1·05–5·61)	0·04
Site		
Axial	1·66 (0·70–3·92)	0·25

Table 4
Summary of the results of the correlation of SOX2 immunoreactivity with metastasis, histological response, tumour volume and relapse.

	Metastasis at diagnosis (n = 141)			Histological response (n = 84)			Tumour volume (n = 136)			Relapse (n = 141)		
	No	Yes	p	Good	Poor	p	<200 ml	≥200 ml	p	No	Yes	p
SOX2												
Low	74 (62·2%)	45 (37·8%)	0·8	57 (79·2%)	15 (20·8%)	1·0	67 (58·3%)	48 (41·7%)	0·23	71 (59·7%)	48 (40·3%)	0·002
High	13 (59·1%)	9 (40·9%)		10 (83·3%)	2 (16·7%)		9 (42·9%)	12 (57·1%)		5 (22·7%)	17 (77·3%)	

independent and strong risk-factor for EwS patients. In fact, SOX2-high expression has been more frequently observed in relapsed tumours, and SOX2-high expression in primary tumours is significantly associated with early relapse in patients with localised disease and no other apparent clinical risk-factors. In line with our findings in EwS, high expression or amplification of *SOX2* has been reported to correlate with poor survival in breast, colorectal, esophageal, laryngeal, endometrial, and ovarian carcinomas [12]. Like in EwS, SOX2 is highly expressed in a subset of patients with lung adenocarcinomas and was found to be an independent predictor of poor survival [27].

Previous reports have suggested that *SOX2* may constitute a direct EWSR1-FLI1 target gene [11,28]. Surprisingly, we found that the vast majority of molecularly confirmed EwS cases do not express SOX2 neither at the mRNA nor the protein level, suggesting that the mode of regulation and the functional role of SOX2 may be more complex than previously thought. Recently, Boulay et al. identified a polymorphic EWSR1-FLI1-bound enhancer-like GGAA-microsatellite near *SOX2*, and demonstrated that epigenetic silencing of this DNA sequence strongly reduced SOX2 expression in EwS cells [28]. In fact, *EWSR1-FLI1* encodes an aberrant transcription factor, which induces many of its target genes by binding to enhancer-like GGAA-microsatellites, whose activity increases with the number of consecutive GGAA-repeats [1,29]. Since the number of GGAA-repeats at such EWSR1-FLI1-bound GGAA-microsatellites is highly variable across individuals [30,31], it is tempting to speculate that the number of GGAA-repeats at the *SOX2*-associated GGAA-microsatellite may contribute to the strong heterogeneity of *SOX2* expression in EwS patients.

In our TMA-cohort, SOX2-high expression showed a correlation with tumour relapse ($p = 0.002$), which likely explains the poor outcome of these patients. In support of our findings, it has been shown in sinonasal carcinomas that patients with *SOX2* amplification have a significantly higher rate of tumour recurrence than those without *SOX2* amplification [32]. However, in our TMA-cohort 38·7% of patients with SOX2-low expression showed relapses, suggesting that in this subset of patients, a different driver might mediate tumour relapse.

Although the correlation between high SOX2 expression and tumour volume was not significant in our TMA-cohort, in line with previous reports regarding EwS [11,33], our *in vitro* and *in vivo* results confirmed a functional role of SOX2 in EwS cell proliferation and tumour growth suggesting that a larger cohort might enable validation of this correlation in EwS patients. In agreement with this, our GSEA in primary EwS indicated that SOX2-high tumours are enriched for gene signatures involved in dedifferentiation, proliferation, and stemness, which may promote tumour relapse. Interestingly, GSEA also revealed that *SOX2* co-expressed genes overlap with genes involved in relapse of malignant melanoma – a tumour of neuroectodermal origin [34] that has also

been proposed for EwS [1]. In support of these data, GSEA of differentially expressed genes upon *SOX2* knockdown in three SOX2-high EwS cell lines demonstrated a significant upregulation of gene signatures involved in differentiation-related processes, such as neurite outgrowth, integrin cell surface interaction, and components of the basement membrane (appendix fig. 5B, appendix table 2).

Despite the exploratory nature of this study, SOX2-high expression may constitute the first promising biomarker for outcome prediction and stratification of high-risk EwS patients with localised disease, which is readily available due to standardised assessments by qRT-PCR and/or IHC. Early identification of such high-risk patients may enable clinicians to apply a closer monitoring and eventually intervene earlier in cases of incipient relapse. In addition, translational and clinical studies aimed at discovering specific therapeutic approaches for this high-risk sub-group of EwS patients could be initiated. Therefore, we recommend validation of these observations in additional prospective studies and experimental elucidation of the precise molecular role of SOX2 in EwS. For prospective studies, we recommend to evaluate the SOX2 expression status by IHC following our staining protocol during routine pathology work-up on diagnostic specimens and to apply the same cut-off as described here (>10% positive nuclei of tumour cells) for classifying samples in SOX2-high and -low, respectively. Since immunohistochemical stains for SOX2 are relatively easy to establish, inexpensive, and straight-forward to evaluate from a pathologist's perspective, we anticipate that this biomarker can be widely used in the clinics. For these reasons, SOX2 will be assessed prospectively in the new iEuroEwing trial led by the Cooperative Ewing Sarcoma Study (CESS) group of the German Society for Paediatric Oncology and Haematology (GPOH) (U. Dirksen personal communication).

Declartation of Competing Interest

The authors declare no conflict of interest.

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Table 5
Summary of the results of the correlation of *SOX2* mRNA with metastasis.

	Metastasis at diagnosis		
	No	Yes	p
SOX2			
Low	45 (71·4%)	18 (28·6%)	0·5
High	8 (61·5%)	5 (38·5%)	

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.08.002.

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