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Prognostic value of intratumoral carbonic anhydrase IX expression in testicular germ cell tumors

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Abstract. Testicular germ cell tumors (TGCTs) represent a highly curable malignancy, however a small proportion of patients fails to be cured with cisplatin-based chemotherapy. Carbonic anhydrase IX (CA IX) is upregulated by hypoxia in several cancer types and correlates with a poor prognosis. The present translational study evaluated expression and prognostic value of CA IX in TGCTs. Surgical specimens from 228 patients with TGCTs were processed by the tissue microarray method and subjected to immunohistochemistry with the M75 monoclonal antibody. CA IX expression was evaluated in tumors vs. adjacent normal testicular tissues and correlated with clinicopathological characteristics and clinical outcome. CA IX expression was detected in 62 (30.2%) of TGCTs compared to 0 (0%) of normal tissue adjacent to testicular tumor (P<0.001). The highest frequency of the CA IX expression was detected in teratoma (39.0%), followed by seminoma (22.7%), yolk sac tumor (22.2%), embryonal carcinoma (11.9%) and choriocarcinoma (7.7%). None of germ cell neoplasias *in situ* (GCNIS) exhibited CA IX expression. Patients without the CA IX tumor expression showed significantly better progression-free survival, but not overall survival, compared to patients with the CA IX expression [hazard ratio (HR), 0.57; 95% CI, 0.32-1.02; P=0.037 and HR, 0.58; 95% CI, 0.29-1.16; P=0.088, respectively]. There was no significant correlation between the CA IX expression and clinicopathological variables. The intratumoral CA IX expression can serve as a prognostic marker in the TGCT patients. These results suggest that activation of the hypoxia-induced pathways may be important in the treatment failure in TGCTs patients.

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Introduction

Testicular germ cell tumors (TGCTs) belong to the most common malignancies among men aged between 20-40 years (1,2). TGCTs are highly curable malignancies and even the majority of metastatic patients may expect to be cured with the first-line cisplatin-based chemotherapy (3,4). Despite the high curative rate, there are ~20-30% of patients who fail to achieve a durable complete remission (3). Salvage chemotherapy based on the standard cisplatin dose and previously non-utilized chemotherapeutic agents or high-dose salvage chemotherapy with autologous stem cell transplantation can induce durable remission in 20-60% of patients with relapsed disease (3,5,6). Current treatment strategies in cisplatin-refractory and relapsed TGCTs patients are insufficient. Thus, novel treatment strategies, including drugs with antitumor activity, as well as novel biomarkers as effective tools for better stratification of patients are required.

The process of tumor development is characterized by rapid proliferation of cancer cells and the expansion of tumor tissue associated with hypoxia in the tumor microenvironment (7). Carbonic anhydrase IX (CA IX) is a zinc metalloenzyme, which catalyzes a reversible hydration of carbon dioxide to

bicarbonate and a proton, and participates in pH regulation, in addition to cell adhesion-migration-invasion (8). Thus, CA IX, localized in the plasma membrane, enables tumor cells to maintain a normal intracellular pH under hypoxic conditions, while concurrently acidifying the extracellular microenvironment. The increased expression of CA IX promotes invasion and metastasis and is associated with treatment resistance in several types of cancer (9-12). Hypoxia and CA IX have been also linked with cancer stem cell properties (13-15). Furthermore, inhibition of angiogenesis is able to generate a hypoxic tumor microenvironment, thereby increasing the population of breast cancer stem cells (16). Thus, an increased proportion of circulating tumor cells derived from tumors that grow under hypoxic conditions may contribute to a poor treatment outcome and increased resistance to chemotherapy (17). Since cancer stem cells in TGCTs resemble embryonic stem cells (9,18), we propose that CA IX, as a mediator of tumor responses to hypoxia, may be involved in the pathogenesis of TGCTs.

The aim of the present translational study was to investigate the CA IX protein expression in TGCTs and to evaluate its potential prognostic role in these patients. We examined the CA IX expression in all histological subtypes of TGCTs, as well as in GCNIS (germ cell neoplasia *in situ*) specimens and in the normal testicular tissue adjacent to testicular tumor.

Patients and methods

Patients. The present translational study (Protocol IZLO1, Chair: M. Mego) involved 228 patients with TGCTs treated from January 2000 to September 2013 in the National Cancer Institute of Slovakia, Bratislava, Slovakia and St. Elisabeth Cancer Institute, Bratislava, Slovakia, with available paraffin embedded tumor tissue specimen and sufficient follow-up clinical data. Patients with a concurrent malignancy other than non-melanoma skin cancer in the previous 5 years were excluded from the study. In all patients, data regarding age, tumor histological subtype, clinical stage and type and number of metastatic lesions were recorded and compared with the CA IX expression. The Institutional Review Board of the National Cancer Institute, Bratislava, Slovakia approved this retrospective study and a waiver of patient consent was granted.

Tumor pathology. Pathological review was conducted at the Department of Pathology, Faculty of Medicine, Comenius University, Bratislava, Slovakia by two pathologists (Z.C. and P.B.) associated with the study.

Diagnosis and tissue samples. Tumor tissue, samples with germ cell neoplasias *in situ* (GCNIS), and normal testicular tissue were evaluated in all cases, when available. The present study included tumor specimens from 228 patients prior to the administration of systemic therapy. Primary testicular tumor specimens were obtained in 205 (89.9%) patients. Biopsies of metastatic sites were available in 23 (10.1%) cases.

The TGCTs were classified according to the World Health Organization criteria (19). Since the normal testicular biopsies from non-cancer patients were not available for our analysis, normal tissue adjacent to testicular tumor was used for evaluation of CA IX expression, as described in previous studies (20-22).

Tissue microarray construction. According to tumor histology, one or two representative tumor areas from each histological subtype of germ cell tumor (from 1-6 cores from each tumor) were identified on haematoxylin and eosin stained sections. Samples from normal testicular tissue and germ cell neoplasia *in situ* were also marked, if present. Sections were matched to their corresponding wax blocks (the donor blocks), and 3-mm diameter cores of the tumor were removed from these donor blocks with the multipurpose sampling tool Harris Uni-Core (Sigma-Aldrich, Steinheim, Germany) and inserted into the recipient master block. The recipient block was cut into 5-μm sections that were transferred to coated slides.

Immunohistochemical staining. Deparaffinized slides were rehydrated in phosphate buffered saline solution (10 mM, pH 7.2). The tissue epitopes were demasked using the automated water bath heating process in Dako PT Link (Dako, Glostrup, Denmark); the slides were incubated in TRIS-EDTA retrieval solution (10 mM TRIS, 1 mM EDTA pH 9.0) at 98˚C for 20 min. This step was introduced because of low intensity of staining when the standard CA IX immunohistochemical staining protocol was used (23). Increasing the concentration, nor the extension of incubation time with the M75 primary antibody, did improve the staining result, however, pretreatment with TRIS-EDTA substantially strengthened the reaction signal. The slides were subsequently incubated for 1 h at room temperature with the monoclonal antibody M75 against the N-terminal domain of the human CA IX protein (23,24) diluted 1:100 in Dako REAL antibody diluent (Dako, Glostrup, Denmark) and immunostained using anti-mouse/anti-rabbit immune-peroxidase polymer (EnVision FLEX/HRP, Dako, Glostrup, Denmark) for 30 min at room temperature, according to the manufacturer's instructions. The reaction was visualized with a diaminobenzidine substrate-chromogen solution (DAB, Dako Cytomation, Denmark) for 5 min, and slides were counter-stained with haematoxylin. The trophoblast staining in human placenta served as positive control. Additional testicular tumors specimens stained with omission of the primary antibody served as negative control.

Immunohistochemistry scoring. Two observes (Z.C. and P.B.) who were blinded to clinicopathological data conducted an independent analysis of the tumor cores. In cases of disagreement, the result was reached by consensus. CA IX expression was stratified as negative or positive (any staining).

Statistical analysis. The patients' characteristics were summarized as mean or median (range) values for continuous variables and frequency (percentage) for categorical variables, respectively. Statistical analysis was performed using non-parametric tests as the distribution of the CA IX expression was significantly different from the normal distribution (Shapiro-Wilk test). Analyses of differences in distributions of CA IX expression between the two groups of patients were performed using the Mann-Whitney U test, whereas Fisher's exact test or the χ^2 test were used when CA IX expression was categorized as ‘absent’ or ‘present’ according to the aforementioned

criteria. Median follow-up period was calculated as a median observation time of all patients and of those still alive at the time of their last follow-up. Progression-free survival (PFS) was calculated from the date of the starting treatment with systemic therapy to the date of progression or death or the date of the last adequate follow-up. Overall survival (OS) was calculated from the date of starting treatment with systemic therapy to the date of death or last follow-up. PFS and OS were estimated using the Kaplan-Meier product limit method and were compared with the log-rank test. A multivariate Cox proportional hazards model for PFS and OS was used to assess differences in outcome on the basis of the CA IX expression in primary tumor and/or biopsy of metastatic site and prognosis according to the IGCCCG (International Germ Cell Collaborative Group) criteria (1997) (19). All presented P-values were two sided. Values of $P<0.05$ were considered to indicate a statistically significant difference. Statistical analyses were performed using NCSS 2007 software developed by Hintze J (2007) (NCSS, LLC, Kaysville, UT, USA) (25).

Results

Patient characteristics. Patients characteristics are summarized in Table I. The mean age of patients enrolled into this study was 30 years (range, 16-67 years). The majority of patients had non-seminomatous primary testicular tumor, and had good prognosis according to the IGCCCG criteria. All patients were treated with cisplatin-based chemotherapy. No extragonadal germ cell tumors were included.

In total, 228 patient specimens were analyzed for CA IX expression using immunohistochemical analysis. CA IX staining was evaluated in 321 tumor specimens from primary testicular tumors (205 patients, Table II), in 23 specimens from metastatic sites sampled post chemotherapy, and in 107 adjacent normal testicular tissues. GCNIS adjacent to testicular tumor was present in 76 patients.

The analyzed cohort of primary testicular tumors included 49 pure seminomas, 79 non-seminomas (57 embryonal carcinomas, 12 yolk sac tumors, 9 teratomas, 1 choriocarcinoma) and 76 mixed germ cell tumors (Table III). Six cases of seminomas were clinically considered as non-seminomas based on positivity of alpha-fetoprotein.

Table I. Patient characteristics (n=228).

Characteristics	No.	%
Age (years)		
Median (range)	30 (16-67)	
Histology		
Seminoma	44	19.3
Non-seminoma	184	80.7
IGCCCG risk group prognosis		
Good	173	75.8
Intermediate	25	11.0
Poor	30	13.2
Sites of metastases		
Retroperitoneum	159	69.7
Mediastinum	23	10.1
Lungs	52	22.8
Liver	12	5.3
Brain	3	1.3
Other	11	4.8
Non-pulmonary visceral metastases	16	7.0
No. of metastatic sites		
0	64	28.1
1	98	43.0
2	34	14.9
>3	32	14.0
Mean (range) of pretreatments markers		
AFP mIU/ml	1168.0 (0.0-60570.0)	
-HCG IU/ml	15980.0 (0.0-929000.0)	
LDH (mkat/l)	11.0 (0.0-88.6)	

-HCG, -human chorionic gonadotropin; AFP, α-fetoprotein; LDH, lactate dehydrogenase; IGCCCG, international germ cell consensus classification group.

Association between CA IX expression and patients/tumor characteristics. CA IX staining of various TGCTs histological subtypes and normal tissue adjacent to testicular tumor is presented in Fig. 1. Whereas normal testicular tissue adjacent to testicular tumor did not show any CA IX staining, CA IX expression was detected in all histological subtypes of TGCTs (Table II). The highest frequency of the CA IX expression was in teratoma samples, with decreasing trend in seminoma, embryonal carcinoma, yolk sac tumor and choriocarcinoma. In choriocarcinomas, the CA IX staining was observed only in one (7.7%) specimen, while no CA IX expression was observed in GCNIS. CA IX expression was detected in 17 seminomas (22.7), 14 embryonal carcinomas (11.9), 8 yolk sac tumors (22.2) and 23 teratomas (39.0) compared to 0 (0.0) of normal testicular tissue adjacent to testicular tumor ($P<0.001$; Table II).

The CA IX staining pattern was largely focal in the tumor tissue, with prevailing membrane positivity in seminomas and embryonal carcinomas and cytoplasmic staining in the other histological subtypes. Mesenchymal cells in teratomas and in the tumor stroma exhibited focal positivity, the intercellular matrix was negative (Fig. 1). Areas of cells neighboring with necrosis did not show increased CA IX expression.

In addition, we analyzed the relationship between the CA IX expression in primary tumors and clinicopathological features (Table IV). We did not find any significant association of the CA IX expression in primary tumors with patients/tumor characteristic, such as tumor histology, IGCCCG risk group, number and localization of metastatic sites and S-stage (Table V). The Spearman's test did not show any significant correlation between the intratumoral CA IX expression and LDH level (Spearman's correlation index, $P=0.216$).

Prognostic value of CA IX. The median follow-up time was 82.3 months (0.3-289.1 months) for all 228 patients and 92.6 months (14.2-289.1 months) for patients still alive. To the

Table II. CA IX expression in different histologic subtypes of the primary germ cell tumors (n=205).

		CA IX expression				
		Absent		Present		
Histological subtype	No.	No.	%	No.	%	P-value
Healthy testis	107	107	100.0	0	0.0	N/A
Testicular germ cell tumors	205	143	69.8	62	30.2	<0.001
Seminoma	75	58	77.3	17	22.7	<0.001
Embryonal carcinoma	118	104	88.1	14	11.9	<0.001
Yolc sac tumor	36	28	77.8	8	22.2	<0.001
Choriocarcinoma	13	12	92.3	1	7.7	0.11
Teratoma	59	36	61.0	23	39.0	<0.001
GCNIS	76	76	100.0	0	0.0	N/A

GCNIS, germ cell neoplasia *in situ*; CA IX, carbonic anhydrase IX.

Table III. Composition of mixed testicular germ cell tumors (n=76).

No. of patients	Histological subtype
22	EC/TER
15	EC/SEM
6	EC/YST/TER
6	YST/TER
5	EC/YST
4	EC/ChC/TER
4	SEM/TER
3	EC/SEM/TER
3	EC/ChC
3	YST/ChC/TER
1	EC/SEM/YST
1	EC/SEM/YST/TER
1	SEM/YST
1	EC/SEM/ChC
1	YST/ChC

EC, embryonal carcinoma; SEM, seminoma; YST, yolk sac tumour; ChC, choriocarcinoma; TER, teratoma.

date of last follow-up, 56 patients (24.6%) experienced disease progression and 38 patients (16.7%) had succumbed. The estimated 2-year and 5-year PFS survival was 80.3% (95% CI, 75.1-85.4%) and 78.3% (95% CI, 72.9-83.7%), while the estimated 2-year and 5-year OS survival was 90.3% (95% CI, 86.5-94.2%) and 85.1% (95% CI, 80.4-89.8%), respectively.

In univariate analysis, patients without CA IX expression in analyzed tumor specimens had significantly better PFS, in contrast to patients with the CA IX expression [hazard ratio (HR), 0.57; 95% CI, 0.32-1.02; P=0.0365; Fig. 2]. Moreover, there was a trend for association between the CA IX expression and OS in this patients' cohort (HR, 0.58; 95% CI, 0.29-1.16; P=0.0876; Fig. 3). Multivariate analysis revealed that CA IX

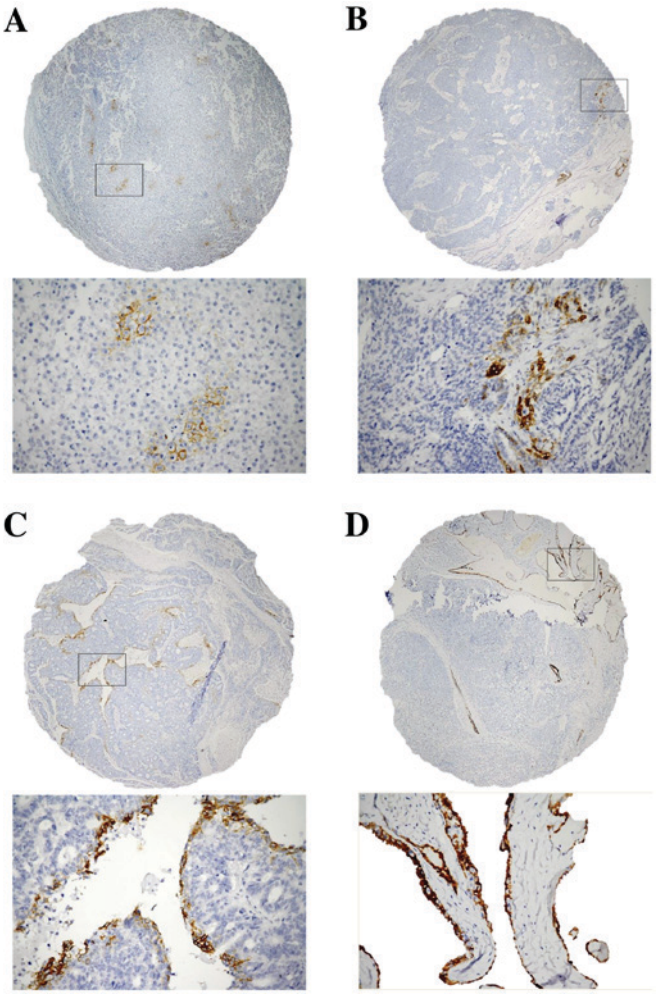

Figure 1. Immunohistochemical detection of CA IX expression in testicular germ cell tumors. (A) Seminoma showed focal moderate membrane CA IX positivity (brown). (B) Yolk sac tumor with focal strong cytoplasmic CA IX positivity. (C) Embryonal carcinoma with focal strong cytoplasmic CA IX positivity. (D) Mature teratoma with strong cytoplasmic CA IX positivity in epithelial component and negativity in mesenchymal component. Magnification, upper x40, lower x400. CA IX, carbonic anhydrase IX.

Table IV. Patient characteristics according to the CA IX expression in the primary tumors (n=205).

		CA IX expression				
		Absent		Present		
Variable	No.	No.	%	No.	%	P-value
All patients	205	143	69.8	62	30.2	N/A
Histology						
Seminoma	40	28	70.0	12	30.0	0.567
Non-seminoma	165	115	69.7	50	30.3	
IGCCCG risk group						
Good/Intermediate prognosis	180	127	70.6	53	29.4	0.325
Poor prognosis	25	16	64.0	9	36.0	
Number of metastatic sites						
0	58	38	65.5	20	34.5	0.841
≥1	147	105	71.4	42	28.6	
Retroperitoneal LN metastases						
Absent	62	40	64.5	22	35.5	0.892
Present	143	103	72.0	40	28.0	
Mediastinal LN metastases						
Absent	187	133	71.1	54	28.9	0.136
Present	18	10	55.6	8	44.4	
Lung metastases						
Absent	160	114	71.3	46	28.8	0.242
Present	45	29	64.4	16	35.6	
Liver						
Absent	194	136	70.1	58	29.9	0.438
Present	11	7	63.6	4	36.4	
Brain						
Absent	204	142	69.6	62	30.4	1.000
Present	1	1	100.0	0	0.0	
Non-pulmonary visceral metastases						
Absent	191	134	70.2	57	29.8	0.423
Present	14	9	64.3	5	35.7	
S-stage						
0-II	186	132	71.0	54	29.0	0.178
III	19	11	57.9	8	42.1	

CA IX, carbonic anhydrase IX; IGCCCG, international germ cell consensus classification group; LN, lymph node.

expression in tumor tissues was associated with PFS independently of the IGCCCG risk group, however this correlation did not reach statistical significance (P=0.0682). Moreover, no significant association was shown between the IGCCCG risk group and the CA IX expression as an independent prognostic factor for overall survival (Table V).

Therefore, exploratory subgroup analysis was performed to reveal a potential subgroup-related prognostic value of CA IX (Table VI). This analysis demonstrated that the tumor CA IX expression correlated with the worse PFS in non-seminoma patients, patients with one or more metastatic lesions and patients with retroperitoneal lymph node metastases. Furthermore, the absence of the CA IX expression in primary tumors was significantly associated with better PFS in patients without lung, brain and non-pulmonary visceral metastases.

Discussion

Carbonic anhydrase IX (CA IX) is a hypoxia-inducible enzyme that is important in cancer development, progression, acidification and metastasis (8). There is increasing evidence that overexpression of CA IX in a variety of cancers correlates with an unfavorable outcome, and is related to a decrease in the progression free survival following successful therapy. Therefore, it is considered as a surrogate tumor biomarker (26).

Table V. Multivariate analysis of the potential prognostic value of CA IX.

	Progression free survival		Overall survival	
Variable	HR (95% CI)	P-value	HR (95% CI)	P-value
CA IX expression in primary tumor high vs. low	1.650 (0.963-2.826)	0.068	1.613 (0.844-3.080)	0.148
IGCCCG risk group poor vs. good/intermediate prognosis	5.260 (3.005-9.209)	<0.001	8.282 (4.286-16.005)	<0.001

CA IX, carbonic anhydrase IX; IGCCCG, international germ cell consensus classification group; HR, hazard ratio; CI, confidence interval.

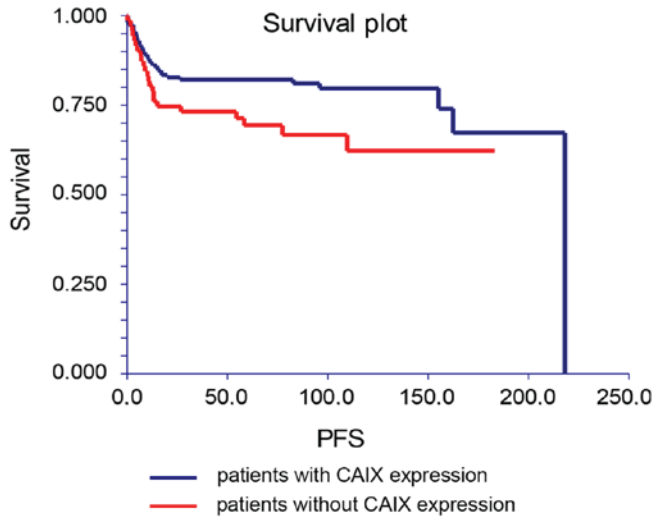

Figure 2. Kaplan-Meier estimates of probabilities of progression-free survival according to CA IX expression in TGCT patients (n=228; HR, 0.57; 95% CI, 0.32-1.02; P=0.037). CA IX, carbonic anhydrase IX; TGCT, testicular germ cell tumors; PFS, progression free survival; HR, hazard ratio; CI, confidence interval.

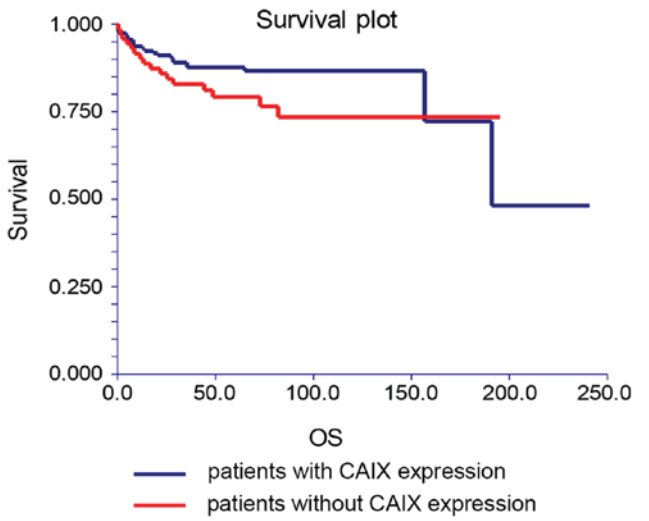

Figure 3. Kaplan-Meier estimates of probabilities of overall survival according to CA IX expression in TGCT patients (n=228; HR, 0.58; 95% CI, 0.29-1.16; P=0.088). CA IX, carbonic anhydrase IX; TGCT, testicular germ cell tumors; OS, overall survival; HR, hazard ratio; CI, confidence interval.

The present translational study demonstrated significantly increased CA IX expression in TGCTs, in contrast to its absence in normal testicular tissue adjacent to germ cell tumors. CA IX expression was demonstrated in all histological subtypes, with the highest expression in teratomas. These findings can be explained by the mesodermal origin of all CA IX expressing cells. Moreover, our results are supported by the detection of CA IX expression in the flat surface epithelium (modified mesothelium) of all male and female genital organs (27). Only one of thirteen choriocarcinoma specimens was positive in CA IX staining. This result is consistent with the study of Donato *et al* (28), where choriocarcinoma tumors were predominately negative in CA IX. In contrast to the results published by Donato *et al* (28), the present study detected CA IX expression not only in teratomas and embryonal carcinomas, but also in seminomas and in yolk sac tumors. On the other hand we identified no CA IX expression in germ cell neoplasia *in situ*. GCNIS represents a precursor lesion for invasive TGCT of the adult testis (29). Thus, we may suppose that CA IX expression does not belong to early events in the pathogenesis of TGCTs. We also failed to detect any significant association between the CA IX expression and the patients/tumor characteristics. However, our data indicated the value of CA IX in the prognosis of progression-free survival, since CA IX expression in analyzed patients tumor specimens correlated with the significantly worse PFS. These findings are in agreement with previous reports on the prognostic value of CA IX in the wide variety of human carcinomas, including upper gastrointestinal cancer, breast cancer, ovarian and cervical cancer, nasopharyngeal cancer, lung and rectal cancer (30-36). The relationship between the CA IX expression and progression-free survival in TGCT patients was confirmed by the subgroup analysis of non-seminoma patients, patients with one or more metastatic site as well as patients with retroperitoneal lymph node metastases. Moreover, the analysis of patients without lung, brain and non-pulmonary visceral metastases showed a similar association. On the other hand, we did not observe any association between the CA IX expression and unfavorable outcome in patients with brain metastases, non-pulmonary visceral metastases and in patients with S-stage 3. It is therefore possible that the poor prognosis of these groups of patients is related to other pathways than that driven by hypoxia. Based on these findings, we propose that CA IX expression, mainly in patients with metastatic

Table VI. Prognostic value of CA IX as an independent indicator in different patient subgroups.

Variable	No.	PFS HR (95% CI)	P-value	OS HR (95% CI)	P-value
Histology					
Seminoma	44	2.46 (0.45-13.48)	0.396	1.00 (0.09-11.00)	0.997
Non-seminoma	184	0.47 (0.25-0.87)	**0.006**	0.54 (0.26-1.12)	0.067
IGCCCG group					
Good prognosis	173	0.68 (0.29-1.62)	0.342	0.56 (0.16-1.94)	0.305
Intermediate prognosis	25	0.49 (0.14-1.72)	0.258	0.88 (0.22-3.53)	0.857
Poor prognosis	30	0.73 (0.29-1.88)	0.499	0.73 (0.27-1.99)	0.518
Number of metastatic sites					
0	64	0.79 (0.14-4.46)	0.756	0.43 (0.05-3.53)	0.317
≥1	164	0.50 (0.27-0.94)	**0.013**	0.56 (0.27-1.19)	0.099
Reroperitoneal LN metastases					
Present	159	0.49 (0.26-0.94)	**0.014**	0.58 (0.27-1.24)	0.120
Absent	69	0.65 (0.14-3.05)	0.536	0.33 (0.05-2.11)	0.141
Mediastinal LN metastases					
Present	23	0.43 (0.13-1.41)	0.161	0.48 (0.12-1.94)	0.303
Absent	205	0.68 (0.35-1.31)	0.210	0.60 (0.26-1.37)	0.1785
Lung metastases					
Present	52	0.82 (0.35-1.92)	0.636	0.69 (0.28-1.68)	0.379
Absent	176	0.48 (0.22-1.05)	**0.035**	0.50 (0.17-1.49)	0.162
Liver metastases					
Present	12	0.30 (0.05-2.01)	0.115	0.32 (0.05-2.05)	0.130
Absent	216	0.60 (0.33-1.12)	0.076	0.62 (0.29-1.32)	0.176
Brain metastases					
Present	3	NA	NA	NA	NA
Absent	225	0.54 (0.30-0.98)	**0.024**	0.53 (0.26-1.08)	0.052
Non-pulmonary visceral metastases					
Present	16	0.52 (0.10-2.64)	0.355	0.53 (0.11-2.68)	0.377
Absent	217	0.56 (0.30-1.04)	**0.041**	0.55 (0.25-1.21)	0.099
S-stage					
0-II	206	0.57 (0.29-1.12)	0.070	0.61 (0.26-1.45)	0.224
III	22	0.62 (0.20-1.91)	0.368	0.51 (0.16-1.67)	0.219

P-values in bold indicate statistically significant differences. CA IX, carbonic anhydrase IX; IGCCCG, international germ cell consensus classification group; HR, hazard ratio; CI, confidence interval; PFS, progression free survival; OS, overall survival; LN, lymph node.

disease but without high-risk features (specifically, patients without brain and non-pulmonary visceral metastases and/or S-stage 3) may serve as prognostic marker of inferior outcome. This suggestion is also supported by the observation, that the hypoxic microenvironment plays a role in inferior outcome and chemoresistance due to increased burden of circulating tumor cells (16). Experimental data suggest that the tumor cells over-express CA IX in order to maintain the intracellular pH and thus preserve their survival in hypoxia (37). The acidification of extracellular space mediated by this mechanism contributes to tumor cell invasion, development of metastases and therefore to worse progression-free survival (38,39). The study has some limitations, including the relative under-representation of choriocarcinoma and yolk sac tumor and the selection of tissue samples into the tissue microarray. For this reason we performed whole tissue section immunohistochemical staining of several TGCT cases, and identified corresponding staining pattern with that in the tissue array. Notably, the TRIS-EDTA pretreatment of the slides considerably increased the sensitivity of CA IX expression detection in the studied tumor specimens.

In conclusion, the present translational study demonstrated significant overexpression of CA IX in TGCTs when compared to normal testicular tissue. We detected for the first time an association between CA IX expression in primary tumor tissue and worse progression-free survival in patients with TGCTs. Higher CA IX expression correlating with inferior outcome was found predominantly in patients with metastatic disease. These results suggest that CA IX expression may serve as an important predictive factor associated with disease recurrence

and poor progression-free survival time in patients with advanced testicular cancer.

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