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RESEARCH

Possible Prognostic Value of BORIS Transcript Variants Ratio in Laryngeal Squamous Cell Carcinomas – a Pilot Study

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Abstract BORIS is a paralog of a highly conserved, multi-functional chromatin factor CTCF. Unlike CTCF, which has been shown to possess tumor-suppressive properties, BORIS belongs to the "cancer/testis antigen" family normally expressed only in germ cells and aberrantly activated in a variety of tumors. The consequences of BORIS expression, relative abundance of its isoforms, and its role in carcinogenesis have not been completely elucidated. It activates transcription of *hTERT* and *MYC*, genes relevant for laryngeal carcinoma progression. In this study, BORIS expression has been analyzed at the transcriptional level by RT-PCR and protein level by semi-quantitative immunohistochemistry in 32 laryngeal squamous cell carcinomas and adjacent non-tumorous tissue. BORIS was detected in 44 % (14/32) laryngeal squamous cell carcinoma samples, while it was detected only in one normal, tumor-adjacent tissue sample. Tree based survival analysis, using the recursive partitioning algorithm *mvpart*, extracted the ratio of relative abundance of BORIS transcript variants containing exon 7 (BORIS 7+) and those lacking exon 7 (BORIS 7−) as an independent prognostic factor associated with disease relapse during a 5-year follow-up period. Patients having BORIS 7+/BORIS 7− ratio ≥1 had a higher rate of disease relapse than patients with BORIS 7+/BORIS 7− ratio <1. Hazard ratio for that group, based on Cox Proportional Hazard Regression, was 3.53. This is the first study analyzing expression of BORIS protein and transcript variants in laryngeal squamous cell carcinoma relative to its possible prognostic value for recurrence and overall survival.

Keywords Laryngeal squamous cell carcinoma · Prognostic value · BORIS · Transcript variant · Immunohistochemistry · RT-PCR

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Introduction

BORIS (Brother of the Regulator of Imprinted Sites; CTCFL) is a paralog of the ubiquitously expressed, multi-functional chromatin factor CTCF (CCCTC-binding factor). Physiological expression of BORIS in spermatocytes coincides with lack of CTCF and the erasure of methylation marks. BORIS expression can be induced by 5-azacytidine in otherwise BORIS-non-expressing cells [1–4]. In malignant cell, aberrantly expressed BORIS competes with CTCF, as both proteins bind to the same DNA motifs due to high homology in their DNA-binding domains, composed of 11 Zn-fingers [1, 5]. However, they significantly differ in NH_2- and COOH-termini, suggesting the recruitment of different protein partners and posttranslational modifications [1, 5–8]. Both proteins have a prominent role in chromatin organization. Contrary to CTCF, BORIS appears to activate transcription [3], although, based on recent data, their role in transcriptional regulation, albeit opposite, is locus-dependent [9]. CTCF is known to use different combinations of Zn-fingers to bind distinct CTCF-binding sites (CBSs) throughout the genome

[10]. Recently, 23 transcript variants resulting in 17 protein isoforms of BORIS have been characterized, differing in the number of Zn-fingers in DNA-binding domain and their NH_2- and COOH-termini [11]. It has been demonstrated that certain BORIS isoforms bind to distinct CBSs. Similarly, BORIS binds more avidly to some CBSs than CTCF, leading to the conclusion that the abundance of those two paralogs and their ratio in the cell are unlikely to be the only determinants of their binding to CBSs. Another recent report showed that BORIS counteracts CTCF's inhibitory effect on transcription of the catalytic subunit of human telomerase reverse transciptase gene (*hTERT*) in testicular and ovarian tumor cells, competing for binding to CBSs and regulating the *hTERT* activity based on their equilibrium [12]. Moreover, the expression of exogenous BORIS in otherwise BORIS non-expressing cells activated transcription of *hTERT* with an increasing number of cell passages and conferred immortality to those cells. It is, therefore, conceivable that regulation of alternative splicing, resulting in presence of different BORIS isoforms, their discriminative binding to distinct CBSs, and the cellular processes affected thereby are extremely complex.

Numerous reports exist on BORIS expression in various tumors [2–4, 12–21], but little is known about its role in laryngeal squamous cell carcinoma (LSCC). Epigenetic changes, arising as a consequence of exposure to carcinogens, play a prominent role in the development of LSSC [21, 22]. Recently, it has been suggested that BORIS involvement in promoter demethylation of putative proto-oncogenes and cancer testis genes, results in their reactivation in head and neck squamous cell carcinoma (HNSCC) and lung cancer [20].

We have explored the expression of several *BORIS* transcript variants and the presence of BORIS protein by immunohistochemistry (IHC) in order to identify its possible prognostic value in LSCC.

Materials and Methods

Tissue Samples

Tissue samples of 32 primary LSCC and adjacent non-tumorous tissue were collected during surgery (from 2001 to 2006) from patients (31 male, one female) treated at the Sestre milosrdnice, University Hospital, Zagreb, Croatia, with patients' written informed consent. The study was approved by the Local Ethics Committee. Histological grading and TNM classification were performed at the Department of Pathology "Ljudevit Jurak." Tumors were graded as well- (G1), moderately- (G2) and poorly-differentiated (G3). The clinical stage was assigned according to recommendations of American Joint Committee on Cancer [23]. The tissues used for total RNA extraction were snap frozen in liquid nitrogen and subsequently stored in the Croatian Tumor Bank at −80 °C until analysis. The tissue sections used for immunohistochemical detection of BORIS were obtained from formalin-fixed, paraffin-embedded (FFPE) tissues.

Total RNA Extraction and RT-PCR

Total RNA was extracted, purified, reversely transcribed and checked for the quality and potential DNA contamination as previously described [24].

Successfully transcribed complementary DNA (cDNA), free of contaminating DNA, was used as template in reverse transcription-polymerase chain reactions (RT-PCR) using *BORIS* primers positioned in exon 5 and exon6/exon7 boundary (UF 5′-CAGGCCCTACAAGTGTAACGACTGCAA-3′ and UR5′-GCATTCGTAAGGCTTCTCACCTGAGTG-3′), which could amplify 13 (A1, A2, A5, A6, B0, B1, B2, B3, B4, B5, C1, C3, and C8) out of 23 reported transcript variants [11]. The 25 μl reaction mixture was composed of deoxyribonucleotide triphosphates (dNTPs) (50 μM each), 1X PrimeSTARBuffer, 6 pmol of each primer and 0.25 U of PrimeSTAR HS DNA polymerase (Takara Bio, Shiga, Japan). Touchdown PCR was performed: in each of the first 13 cycles, annealing temperature was decreased by 1 °C starting from 68 °C, and followed by 25 cycles with the annealing temperature at 55 °C. The PCR products (271 bp) were visualized on 2 % agarose gel containing SYBR® Safe DNA Gel Stain.

The presence of alternatively spliced *BORIS* transcripts was detected with primers designed to amplify the region between exons 5 and 9 (SF 5′- TCACCAGTGGAGAACT CGTCCG-3′ and SR 5′- TGCAAGAAAGGCAGGTGAAT GGT-3′), which amplify, with exception of C8, all previously mentioned transcript variants and three additional: A3, C4, and C6 which differ in the Zn-finger coding region (Fig. 3a). The 25 μl reaction mixture was composed of dNTPs (50 μM each), 1X GeneAmp PCR Buffer containing 1.5 mM $MgCl_2$ (Applied Biosystems, Foster City, CA), 6 pmol of each primer and 0.25 U of Gold AmpliTaq DNA polymerase (Applied Biosystems). Touchdown PCR was performed: in each of the first 10 cycles, annealing temperature was decreased by 1 °C starting from 68 °C, and followed by 32 cycles with the annealing temperature at 58 °C. PCR products (591 bp, 441 bp or 159 bp) were visualized on 2 % agarose gel containing ethidium bromide, excised from the gel, purified and sequenced.

Immunohistochemical Staining and Assessment of BORIS

Immunohistochemical analysis was done on FFPE tissue sections of LSCCs containing adjacent non-tumorous tissues. Prior to IHC staining, antigen retrieval by boiling sections in 0.01 M citrate buffer, pH 6.0 was performed. BORIS was detected using anti-BORIS antibody (ab18337, Abcam, Cambridge, UK) and Vectastain Elite ABC standard kit (Vector

Laboratories, Burlingame, CA) according to the manufacturer's instructions. Immunohistochemical staining was scored as previously described for detection of BORIS [14, 18]. BORIS-positive sections were divided into four categories according to the percentage of BORIS-positive cells (<10 %=1, 10–50 %=2, >50–80 %=3, >80 %=4). The intensity of staining was graded from 0 (undetectable staining) to 3 (strong immunostaining). Immunoreactivity score (IRS) was calculated by multiplying the number of the category and staining intensity grade.

Statistical Analysis

Fisher's exact test was used to analyze the distribution of BORIS according to various clinico-pathological parameters and the disease outcome, i.e., relapse or survival. All *p*-values were two-sided and the threshold was set at 0.05. BORIS immunoreactivity with IRS ≥ 1 was categorized as positive. For mRNA expression studies, the samples with detectable PCR product after 42 PCR cycles were considered positive, regardless of the transcript variant amplified. The ratio of *BORIS* transcript variants was calculated based on the gel densitometry (EZQuant software) of bands corresponding to *BORIS*(7+) and *BORIS* (7−) amplicons. Survival data were available for all 32 patients.

Relapse-free survival was calculated from the date of first surgery to the date of clinical or pathological recurrence. Overall survival was calculated from the date of first surgery to the date of death. Survival analysis was made using the *mvpart* library inside statistical software R [25, 26]. The algorithm constructs subgroups that are internally as homogeneous as possible with regard to the outcome (relapse) and externally as separated as possible. In this way the method leads directly to prognostic subgroups defined by the potential prognostic factors. This is achieved by a recursive tree building algorithm. The final result is a binary tree with a set of patients, a splitting rule, and the hazard ratio value at each interior node with the respect to the starting node [27].

Results

Expression of BORIS in LSCC

Thirty-two samples of laryngeal carcinoma and adjacent non-tumorous tissue were analyzed by IHC and RT-PCR for presence of BORIS protein and its transcripts. BORIS was detected by both methods in 14/32 (43.75 %) tumor samples. Qualitatively, the expression of BORIS mRNA in LSCC samples, as detected with BORIS primers UF and UR, correlated well with the presence of BORIS protein. However, quantitative correlation was not observed.

Strong membranous and cytoplasmic BORIS immunostaining was present in LSCC samples. Staining was confined to tumorous squamous epithelium and to morphologically untransformed seromucinous glandular tissue (Fig. 1a). In several tumors of grades 1 and 2, BORIS signal was confined only to seromucinous glandular tissue. It was not detected in normal or metaplastic squamous epithelium, albeit a weak cytoplasmatic staining was observed in non-tumorous tissue seromucinous glands (Fig. 1b). Only one sample of non-tumorous tissue was BORIS-positive at transcript level.

The amplification of cDNA with primers SF and SR resulted in full size PCR product (591 bp long) and two PCR products that were 150 bp or 432 bp shorter, missing exon 7 and exons 6–8, respectively (Fig. 2a), as confirmed by sequencing of these PCR products (Fig. 2b). The densitometry of PCR products containing exons 5–9 (BORIS 7+) or lacking exon 7/exons 6–8 (BORIS 7−) allowed us to evaluate the prognostic significance of those two groups of transcript variants. Most BORIS-positive tumors contained both groups of transcript variants, but their ratio differed considerably (Fig. 3).

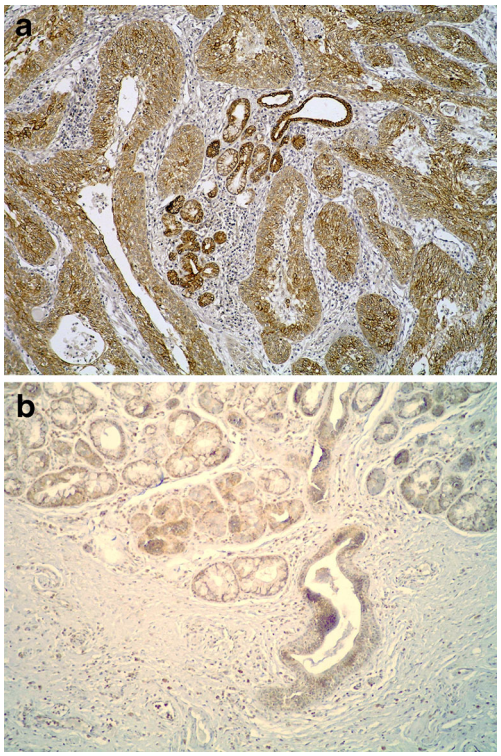

Fig. 1 BORIS IHC staining. **a** Squamous cell carcinoma of the larynx. Strong membranous and cytoplasmatic immunopositivity of BORIS in tumor cells. **b** Moderate cytoplasmatic BORIS expression in normal seromucinous glands of the larynx. Sections were counterstained with hematoxylin and secondary antibodies were revealed with 3, 3′-diaminobenzidine (*brown*). Original magnification x100

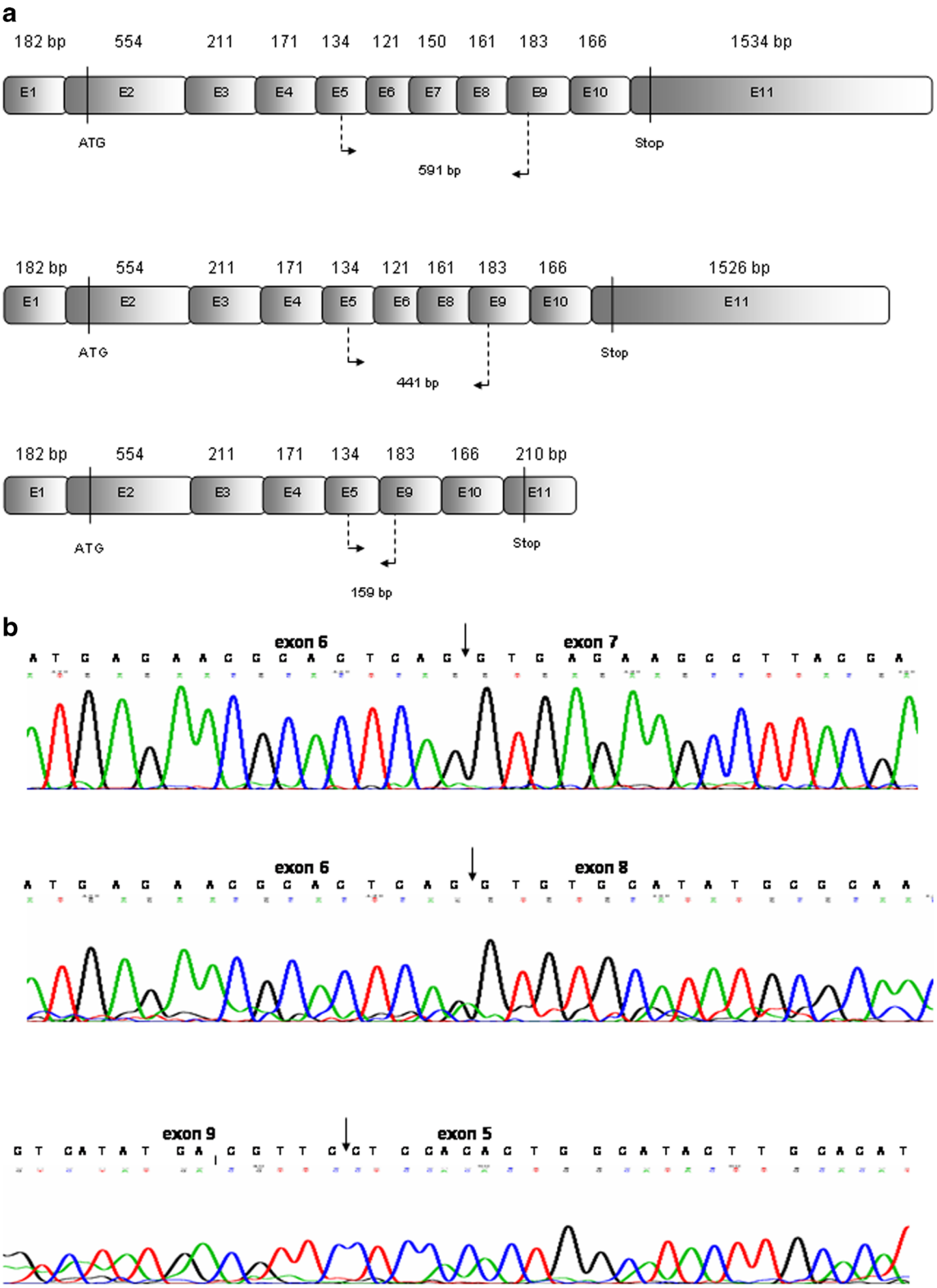

Fig. 2 **a** *BORIS* mRNA and primer design. Exons of *BORIS* mRNA (ENSEMBL # ENST00000243914, CTCFL-001; # ENST00000429804, CTCFL-205; # ENST00000432255, CTCFL-206) are depicted as boxes with corresponding sizes in base pairs above. *Arrows* indicate primers used to amplify alternatively spliced transcript variants with the sizes of corresponding PCR products in base pairs. **b** Chromatogram of the PCR product of transcript variant containing exons 5–9, transcript variant lacking exon 7 and transcript variant lacking exons 6–8. *Arrows* indicate exon boundaries

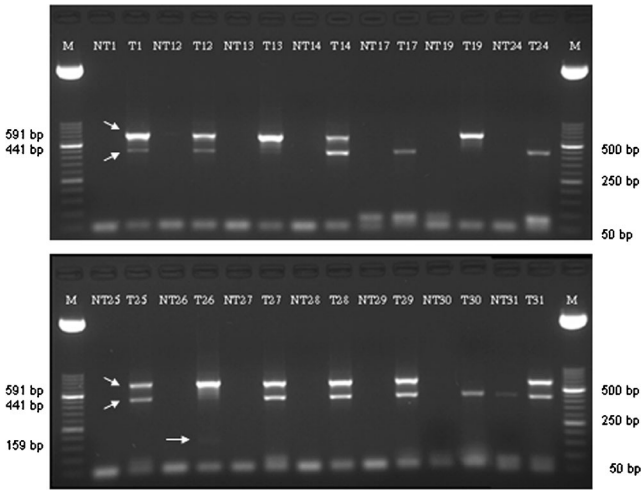

Fig. 3 Expression of BORIS transcript variants in BORIS positive LSCC and adjacent non-tumorous tissue samples. M designates DNA molecular weight marker XIII (50–750 bp). N designates non-tumorous tissue with corresponding sample number. T designates tumor tissue with corresponding sample number. *Arrows* point to bands corresponding to amplicons 591, 441 and 159 bp

Correlation Between BORIS Expression and Clinicopathological Characteristics in LSCC

The distribution of BORIS immunopositivity and BORIS 7+/BORIS 7− ratio according to clinico-pathological characteristics was analyzed by Fisher's exact test.

There was statistically significant relationship between BORIS positivity and the patient's age of ≤59 years (p=0.011), and poor differentiation of tumors (p=0.01). None of the BORIS-negative tumors was in the group of G3 tumors. There was no significant relationship between BORIS immunoreactivity and T- classification, lymph node involvement or clinical stage (Table 1).

The results of analysis of total BORIS transcripts (primers UF and UR) were comparable with those obtained by immunohistochemical analysis, i.e., all tumor samples positive at transcript level were also positive by immunohistochemistry (n=14). However, when the ratio of relative abundance of transcripts, BORIS(7+)/BORIS(7−)≥1 vs. BORIS(7+)/BORIS(7−)<1 or BORIS negative was analyzed, statistical significance was observed between the ratio ≥1 and poor differentiation (p=0.034) as well as lymph node involvement (p=0.006) but not with age, clinical stage or T-classification (Table 1).

Correlation Between BORIS Expression and Disease Outcome

During the follow-up period, regional relapses were observed in 8/32 patients (25 %). By the end of the study, 5/32 (16 %) patients had died.

The distribution of BORIS immunopositivity and BORIS 7+/BORIS 7− ratio relative to disease relapse and survival is shown in Table 2.

There was statistically significant relationship between BORIS positivity and lethal outcome within 5 years of surgery (p=0.0099), i.e., none of the patients with BORIS-negative tumors died within this period. There was no statistically significant relationship between BORIS immunopositivity and the disease relapse within 5 years post surgery (p=0.096).

When comparing groups of patients that differ in BORIS 7+/BORIS 7− ratio (<1 vs. ≥1), there was statistically significant relationship between BORIS 7+/BORIS 7− ratio ≥1 and both relapse of the disease (p=0.0000308) and lethal outcome (p=0.000029) within 5 years post surgery.

Survival Analysis

Survival analysis using *mvpart* algorithm extracted the ratio of relative abundance of transcripts, BORIS(7+)/BORIS(7−)≥1 vs. BORIS(7+)/BORIS(7−)<1 or BORIS negative as the independent prognostic factor associated with disease relapse (Fig. 4). Only two relapses were observed in the group of patients with the BORIS 7+ / BORIS 7− ratio < 1 (n=26). On the contrary, relapses of the disease were observed in all patients with BORIS 7+ /BORIS 7− ratio ≥ 1 (n=6). Additionally, the extracted prognostic subgroups were compared using univariate Cox regression, log-rank and Wilcoxon tests [28]. The difference between subgroups in relapse rates was statistically significant by all methods, at a p level of 0.05 (Table 3). Kaplan-Meier survival estimation and log-rank test, using this ratio as categorical covariate (≥1 vs. <1 or BORIS negative), showed a significant relationship between BORIS (7+)/ BORIS (7−) ratio ≥1 and relapse of the disease within 5 years follow-up (Fig. 5). Hazard ratio (univariate Cox regression) for patients with BORIS 7+ /BORIS 7− ratio ≥ 1 was 3.53 (95 % confidence interval 1.37–8.51) with the respect to the starting node, i.e., all patients.

Discussion

Stratified epithelium of the larynx is subject to a variety of environmental, genotoxic insults with chronic alcohol consumption, cigarette smoking and HPV being the major risk factors for developing LSCC [29]. Global DNA hypomethylation is one of the consequences of exposure to these risk factors [30, 31]. Considering that BORIS expression can be induced by demethylating agents, we have evaluated BORIS expression in, often, alcohol and smoking related LSCC.

As demonstrated immunohistochemically, BORIS was expressed only in tumor tissue and not in adjacent non-tumorous tissue except for weak staining of seromucinous glands. The used antibody was raised against synthetic peptide

Table 1 BORIS immunostaining and BORIS 7+/BORIS 7− ratio according to clinico-pathological characteristics in 32 squamous laryngeal carcinoma patients

	Tumor immunostaining			BORIS 7+/ BORIS 7− ratio		
				BORIS−		
	BORIS−	BORIS +	*p*-value[a]	or ratio <1	ratio ≥1	*p*-value [a]
Age (years)						
≤59	5 (15.625 %)	11 (34.375 %)		11 (34.375 %)	5 (15.625 %)	
≥60	13 (40.625 %)	3 (9.375 %)	0.011	15 (46.875 %)	1 (3.125 %)	0.172
Histopathological grading						
G1-G2	18 (56 %)	9 (28 %)		24 (75 %)	3 (9.375 %)	
G3	0 (0 %)	5 (16 %)	0.01	2 (6.25 %)	3 (9.375 %)	0.034
Clinical stage						
I-II	3 (9.375 %)	2(6.25 %)		5 (15.625 %)	0 (0 %)	
III-IV	15 (46.875 %)	12 (37.5 %)	1.00	21 (65.625 %)	6 (18.75 %)	0.555
T-classification						
1–2	6 (19 %)	6 (19 %)		10 (31.25 %)	2 (6.25 %)	
3–4	12 (37 %)	8 (25 %)	0.718	16 (50 %)	4 (12.5 %)	1.00
Lymph node involvement						
N0	12 (37 %)	5 (16 %)		17 (53.125 %)	0 (0 %)	
N+	6 (19 %)	9 (28 %)	0.072	9 (28.125 %)	6 (18.75 %)	0.006

[a] Fisher's exact test. *p*-value <0.05 was considered significant

derived from within residues 1–100 of human BORIS, and may not be suitable for detection of all BORIS protein isoforms. Hence, the detected number of BORIS-positive samples could be an underestimate of the actual number of BORIS-positive samples. We have chosen an immunoreactivity score method for evaluation of IHC staining for several reasons: 1) it has been used for scoring the immunohistochemical staining of BORIS in published works by other authors, 2) very little data are available on BORIS detection by immunohistochemistry, 3) the level and distribution (both tissue and subcellular) of different BORIS isoforms have been largely unexplored, and 4) the lack of the antibodies able to discriminate between BORIS isoforms corresponding to transcript variants detected in laryngeal carcinoma samples. Due to all these reasons, IHC was used only for detection of the presence or the absence of BORIS (not knowing the isoform composition and ratio) in the samples in order to get an insight of its tissue and subcellular localization. Accordingly, we have classified samples as BORIS positive or negative. The greater precision of the BORIS quantification, which would be possible by employing the H-score method [32] for evaluating IHC staining, would be preferred and meaningful approach once antibodies specific for different BORIS isoforms become available. The mainly cytoplasmatic detection of BORIS in our study is in agreement with declarations given by the antibody manufacturer based on antibody testing on breast carcinoma tissue. However, both cytoplasmatic and nuclear localization of BORIS have been reported in breast tumors

Table 2 BORIS immunostaining and BORIS 7+/BORIS 7− ratio according to clinical outcome within 5-years follow-up of 32 squamous laryngeal carcinoma patients

	Tumor immunostaining			BORIS 7+/ BORIS 7− ratio		
				BORIS−		
	BORIS−	BORIS +	*p*-value[a]	or ratio <1	ratio ≥1	*p*-value [a]
Disease relapse						
Relapse	2 (6.25 %)	6 (18.75 %)		2 (6.25 %)	6 (18.75 %)	
No relapse	16 (50 %)	8 (25 %)	0.096	24 (75 %)	0 (0 %)	0.0000308
Survival						
Lethal outcome	0 (0 %)	5 (15.625 %)		0 (0 %)	5 (15.625 %)	
Alive	18 (56.25 %)	9 (28.125 %)	0.0099	26 (81.25 %)	1 (3.125 %)	0.000029

[a] Fisher's exact test. *p*-value <0.05 was considered significant

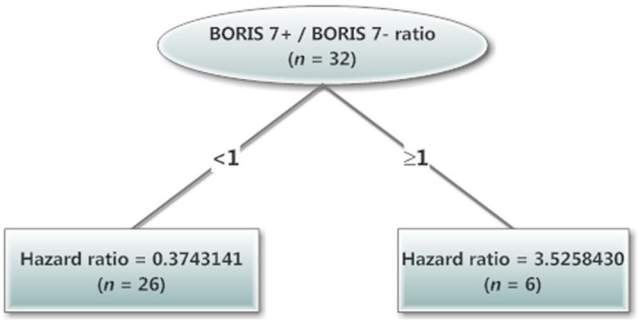

Fig. 4 Survival tree constructed using *mvpart* algoritm, with BORIS 7+ / BORIS 7− ratio as the only predictor of the disease relapse

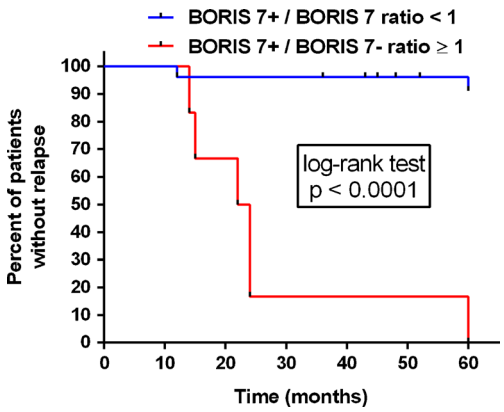

Fig. 5 Kaplan-Meier estimation and log-rank test for the prognostic subgroups BORIS (7+)/ BORIS (7−) ≥1 and BORIS (7+)/ BORIS (7−) <1

[18]. In human urogenital malignancies, the signal was dependent on the developmental stage of the cells: cytoplasmatic immunostaining of BORIS has been shown in spermatogonia, while it was nuclear in spermatocytes [4]. In the same study, in addition to cytoplasmatic staining in prostate cancer tissue, BORIS was also detected in the cytoplasm of basal cells and was membrane-associated in secretory epithelial cells in normal prostate tissue. The identity of BORIS isoform/s in normal glandular tissue, detected by IHC in this study is not known. Its role in normal tissue outside of testis has not been established, although a regulatory role in the cell cycle has been suggested [33]. The much stronger staining of morphologically normal glands within a tumor mass than that of glandular ducts within adjacent normal tissue may well be a result of molecular changes that had taken place in morphologically untransformed secretory epithelium. We observed previously that changes on the molecular level, namely loss of imprinting of insulin-like growth factor 2, occur in the morphologically untransformed, tumor adjacent tissue [34].

So far, there are no available data on possible nucleocytoplasmic shuttling of BORIS, despite an important role of this process for the function of many regulatory proteins during spermatogenesis [35]. Cytoplasmatic localization of BORIS seems to be discordant with a recent report, showing that all BORIS isoforms are localized exclusively in the nuclei of HEK293T, NHDF and HCT-15 cells [11] although this discrepancy may stem from the experimental approach used in the cited study. The proposed role of BORIS, through its association with DNA, does not seem to be consistent with its cytoplasmatic localization. Although many BORIS isoforms contain a nuclear localization sequence, there is still an unexplored possibility that some of those isoforms interact with protein partners in the cytoplasm and may not enter the nucleus. One of the identified BORIS-interacting proteins is BAT3 [36], predominantly localized in cytoplasm in various normal mammalian cells [37]. Although human BAT3 contains a nuclear localization sequence [38], its nuclear localization appears to be dependent upon malignant transformation [39]. It is, thus, conceivable that DNA-bound BORIS may serve as a "platform" to which BAT3 binds and is retained in the nucleus [36].

The significant correlation between BORIS immunoreactivity and age ≤59 years (p=0.011) shown in our study has not been observed in breast cancer patients [18], probably due to the differences in gender-related, age-related and tissue-specific epigenetic changes, most notably DNA methylation changes, that occur during carcinogenesis and tumor progression at those two unrelated anatomical sites [40]. The significant relationship between BORIS immunopositivity and poor differentiation (p=0.01) has not been observed in breast cancer [18] and we do not exclude that our results may be biased by a small number of poorly differentiated tumors (G3, n=5) which were all BORIS-positive at both transcriptional and protein level.

Despite weak BORIS immunostaining of basal cells in seromucinous glands of some non-tumorous tissues, BORIS transcripts were detected only in one of these samples. The reason may be a very low level of BORIS transcripts and/or the anatomical location of primary tumor influencing the sampling of the adjacent non-tumorous tissue from locations other than the false vocal cord and the ventricle where seromucinous glands are abundant. The low abundance of BORIS transcripts in some tumors may stem from uneven distribution of BORIS positive cells within a heterogeneous population of cells, indicating its presence in a certain cell type and/or particular stage of tumor development [11]. The expression of BORIS transcripts was in agreement with its expression at the protein level. We initially used the primers UF and UR that have been widely used in BORIS expression

Table 3 The comparison of prognostic subgroups (BORIS 7+ / BORIS 7− ratio <1 and BORIS 7+ / BORIS 7− ratio ≥ 1) extracted using *mvpart* algorithm

Analysis	Results
Univariate Cox Regression	p=0.0083
Log-rank test	p<0.0001
Wilcoxon test	p<0.0001

studies. An emerging awareness of the existence of multiple BORIS transcript variants [41, 11] and a report on naturally occurring transcript variant/s lacking exon 7 in human testis [42], prompted us to construct another primer pair (SF and SR), specifically designed to amplify both exon 7-containing and exon 7-lacking transcript variants. The predominance of variant/s BORIS (7+) significantly correlated to poor tumor differentiation (p=0.034) and lymph node involvement (p= 0.006). However, while very promising, these results need to be taken with caution due to the relatively small number of analyzed samples. There is no doubt that many more tumors (first of all, those smoking-related) need to be analyzed, focusing on specific BORIS isoforms.

There is no information on the distribution/functional consequence of BORIS transcript variants in primary tumors of any origin. Likewise, considering the presence of BORIS protein both in the cytoplasm and nuclei of tumor cells, it would be necessary to decipher the cellular compartmentalization of BORIS isoforms and identify their protein binding partners.

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References

1. Loukinov DI, Pugacheva E, Vatolin S, Pack SD, Moon H, Chernukin I, Mannan P, Larsson E, Kanduri C, Vostrov AA, Cui H, Niemitz EL, Rasko JE, Docquier FM, Kistler M, Breen JJ, Zhuang Z, Quitschke WW, Renkawitz R, Klenova EM, Feinberg AP, Ohlsson R, Morse HC 3rd, Lobanenkov VV (2002) BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma. Proc Natl Acad Sci U S A 99:6806–6811
2. Vatolin S, Abdullaev Z, Pack SD, Flanagan PT, Custer M, Loukinov DI, Pugacheva E, Hong JA, Morse H 3rd, Schrump DS, Risinger JI, Barrett JC, Lobanenkov VV (2005) Conditional expression of the CTCF-paralogous transcriptional factor BORIS in normal cells results in demethylation and derepression of MAGE-A1 and reactivation of other cancer-testis genes. Cancer Res 65:7751–7762
3. Hong JA, Kang Y, Abdullaev Z, Flanagan PT, Pack SD, Fischette MR, Adnani MT, Loukinov DI, Vatolin S, Risinger JI, Custer M, Chen GA, Zhao M, Nguyen DM, Barrett JC, Lobanenkov VV, Schrump DS (2005) Reciprocal binding of CTCF and BORIS to the NY-ESO-1 promoter coincides with derepression of this cancer-testis gene in lung cancer cells. Cancer Res 65:7763–7774
4. Hoffmann MJ, Müller M, Engers R, Schulz WA (2006) Epigenetic control of CTCFL/BORIS and OCT4 expression in urogenital malignancies. Biochem Pharmacol 72:1577–1588
5. Klenova EM, Morse HC, Ohlsson R, Lobanenkov VV (2002) The novel BORIS+CTCF gene family is uniquely involved in epigenetics of normal biology and cancer. Semin Cancer Biol 12:399–414
6. Ohlsson R, Renkawitz R, Lobanenkov V (2001) CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. Trends Genet 17:520–527
7. Klenova E, Ohlsson R (2005) Poly(ADP-ribosyl)ation and epigenetics: is CTCF PARt of the plot? Cell Cycle 4:96–101
8. Jelinic P, Stehle J-C, Shaw P (2006) The testis-specific factor CTCFL cooperates with the protein methyltransferase PRMT7 in H19 imprinting control region methylation. PLoS Biol 4:1910–1922
9. Fiorentino FP, Macaluso M, Miranda F, Montanari M, Russo A, Bagella L, Giordano A (2011) CTCF and BORIS regulate Rb2/p130 gene transcription: a novel mechanism and a new paradigm for understanding the biology of lung cancer. Mol Cancer Res 9:225–233
10. Filipova GN, Fagerlie S, Klenova EM, Myers C, Dehner Y, Goodwin G, Neiman PE, Collins SJ, Lobanenkov VV (1996) An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-myc oncogenes. Mol Cell Biol 16:2802–2813
11. Pugacheva EM, Suzuki T, Pack SD, Kosaka-Suzuki N, Yoon J, Vostrov AA, Barsov E, Strunnikov AV, Morse HC III, Loukinov D, Lobanenkov V (2010) The structural complexity of the human BORIS gene in gametogenesis and cancer. PLoS One 5:e13872
12. Renaud S, Loukinov D, Alberti L, Vostrov A, Kwon Y-W, Bosman FT, Lobanenkov V, Benhattar J (2011) BORIS/CTCFL-mediated transcriptional regulation of the hTERT telomerase gene in testicular and ovarian tumor cells. Nucleic Acids Res 39:862–873
13. Ulaner GA, VuTH LT, Hu J-F, Yao X-M, Yang Y, Gorlick R, Meyers P, Healey J, Ladanyi M, Hoffman AR (2003) Loss of imprinting of IGF2 and H19 in osteosarcoma is accompanied by reciprocal methylation changes of a CTCF-binding site. Hum Mol Genet 12:535–549
14. D'Arcy V, Abdullaev ZK, Pore N, Docquier F, Torrano V, Chernukin I, Smart M, Farrar D, Metodiev M, Fernandez N, Richard C, Delgado DM, Lobanenkov V, Klenova E (2006) The potential of BORIS detected in the leukocytes of breast cancer patients as an early marker of tumorigenesis. Clin Cancer Res 12:5978–5986
15. Looijenga LH, Hersmus R, Gillis AJ, Pfundt R, Stoop HJ, van Gurp RJ, Veltman J, Beverloo HB, van Drunen E, van Kessel AG, Pera RR, Schneider DT, Summersgill B, Shipley J, McIntyre A, van der Spek P, Schoenmakers E, Oosterhuis JW (2006) Genomic and expression profiling of human spermatocytic seminomas: primary spermatocyte as tumorigenic precursor and DMRT1 as candidate chromosome 9 gene. Cancer Res 66:290–302
16. Risinger JI, Chandramouli GVR, Maxwell GL, Custer M, Pack S, Loukinov D, Aprelikova O, Litzi T, Schrump DS, Murphy SK, Berchuck A, Lobanenkov V, Barrett JC (2007) Global expression analysis of cancer/testis genes in uterine cancers reveals a high incidence of BORIS expression. Clin Cancer Res 13:1713–1719
17. Woloszynska-Read A, James SR, Link PA, Yu J, Odunsi K, Karpf AR (2007) DNA methylation-dependent regulation of BORIS/CTCFL expression in ovarian cancer. Cancer Immun 7:21
18. D'Arcy V, Pore N, Docquier F, Abdullaev ZK, Chernukhin I, Kita GX, Rai S, Smart M, Farrar D, Pack S, Lobanenkov V, Klenova E (2008) BORIS, a paralogue of the transcription factor, CTCF, is aberrantly expressed in breast tumors. Br J Cancer 98:571–579
19. Kholmanskikh O, Loriot A, Brasseur F, De Plaen E, De Smet C (2008) Expression of BORIS in melanoma: lack of association with MAGE-A1 activation. Int J Cancer 122:777–784
20. Smith IM, Glazer CA, Mithani SK, Ochs MF, Sun W, Bhan S, Vostrov A, Abdullaev Z, Lobanenkov V, Gray A, Liu C, Chang SS, Ostrow KL, Westra WH, Begum S, Dhara M, Califano J (2009) Coordinated activation of candidate proto-oncogenes and cancer testes antigens via promoter demethylation in head and neck cancer and lung cancer. PLoS One 4:e4961
21. Smiraglia DJ, Smith LT, Lang JC, Rush LJ, Dai Z, Schuller DE, Plass C (2003) Differential targets of CpG island hypermethylation in primary and metastatic head and neck squamous cell carcinoma (HNSCC). J Med Genet 40:25–33
22. Schmezer P, Plass C (2008) Epigenetic aspects in carcinomas of the head and neck. HNO 56:594–602

23. Larynx D et al (2010) In: Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A (eds) AJCC cancer staging manual, 7th edn. Springer, New York, pp 57–62
24. Grbeša I, Ivkić M, Pegan B, Gall-Trošelj K (2006) Loss of imprinting and promoter usage of the IGF2 in laryngeal squamous cell carcinoma. Cancer Lett 238:224–229
25. rpart by Terry M Therneau, Beth Atkinson. R port of rpart by Brian Ripley Rripley@stats.ox.ac.uk>. Some routines from vegan – Jari Oksanen < jari.oksanen@oulu.fi > Extensions and adaptations of rpart to mvpart by Glenn De'ath. (2013) mvpart: Multivariate partitioning. R package version 1.6-1. http://CRAN.R-project.org/package=mvpart. Accessed 15 Jan 2014
26. R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/ Accessed 15 Jan 2014
27. Schumacher M, Holländer N, Schwarzer G, Willi Sauerbrei W (2006) Prognostic factor studies. In: Crowley J, Ankerst DP (eds) Handbook of statistics in clinical oncology, 2nd edn. Chapman & Hall/CRC, Boca Raton, pp 289–333
28. Liu X (2013) Survival analysis. Wiley, Chichester
29. Ragin CC, Modugno F, Gollin SM (2007) The epidemiology and risk factors of head and neck cancer: a focus on human papillomavirus. J Dent Res 86:104–114
30. Pöschl G, Stickel F, Wang XD, Seitz HK (2004) Alcohol and cancer: genetic and nutritional aspects. Proc Nutr Soc 63:65–71
31. Flatley JE, McNeir K, Balasubramani L, Tidy J, Stuart EL, Young TA, Powers HJ (2009) Folate status and aberrant DNA methylation are associated with HPV infection and cervical pathogenesis. Cancer Epidemiol Biomarkers Prev 18:2782–2789
32. McCarty KS Jr, Szabo E, Flowers JL, Cox EB, Leight GS, Miller L, Konrath J, Soper JT, Budwit DA, Creasman WT, Seigler HF, McCarty KS Sr (1986) Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. Cancer Res 46:4244s–4248s
33. Rosa-Garrido M, Ceballos L, Alonso-Lecue P, Abraira C, Delgado MD, Gandarillas A (2012) A cell cycle role for the epigenetic factor CTCF-L/BORIS. PLoS One 7:e39371
34. Grbesa I, Marinkovic M, Ivkic M, Kruslin B, Novak-Kujundzic R, Pegan B, Bogdanovic O, Bedekovic V, Gall-Troselj K (2008) Loss of imprinting of IGF2 and H19, loss of heterozygosity of IGF2R and CTCF, and helicobacter pylori infection in laryngeal squamous cell carcinoma. J Mol Med (Berl) 86:1057–1066
35. Hogarth C, Itman C, Jans DA, Loveland KL (2005) Regulated nucleocytoplasmic transport in spermatogenesis: a driver of cellular differentiation? BioEssays 27:1011–1025
36. Nguyen P, Bar-Sela G, Sun L, Bisht KS, Cui H, Kohn E, Feinberg AP, Gius D (2008) BAT3 and SET1A form a complex with CTCFL/BORIS to modulate H3K4 histone dimethylation and gene expression. Mol Cell Biol 28:6720–6729
37. Ozaki T, Hanaoka E, Naka M, Nakagawara A, Sakiyama S (1999) Cloning and characterization of rat BAT3 cDNA. DNA Cell Biol 18:503–512
38. Manchen ST, Hubberstey AV (2001) Human Scythe contains a functional nuclear localization sequence and remains in the nucleus during staurosporine-induced apoptosis. Biochem Biophys Res Commun 287:1075–1082
39. Tsukahara T, Kimura S, Ichimiya S, Torigoe T, Kawaguchi S, Wada T, Yamashita T, Sato N (2009) Scythe/BAT3 regulates apoptotic cell death induced by papillomavirus binding factor in human osteosarcoma. Cancer Sci 100:47–53
40. Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. Oncogene 21:5400–5413
41. Renaud S, Pugacheva EM, Delgado MD, Braunschweig R, Abdullaev Z, Loukinov D, Benhattar J, Lobanenkov V (2007) Expression of the CTCF-paralogous cancer-testis gene, brother of the regulator of imprinted sites (BORIS), is regulated by three alternative promoters modulated by CpG methylation and by CTCF and p53 transcription factors. Nucleic Acids Res 35:7372–7388
42. Hines WC, Bazarov AV, Mukhopadhyay R, Yaswen P (2010) BORIS (CTCFL) is not expressed in most human breast cell lines and high grade breast carcinomas. PLoS One 5:e9738