Genome-wide copy number profiling using a 100K SNP array reveals novel disease-related genes *BORIS* and *TSHZ1* in juvenile angiofibroma

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Abstract. Juvenile angiofibroma (JA) is a unique fibrovascular tumor, which is almost exclusively found in the posterior nasal cavity of adolescent males. Although histologically classified as benign, the tumor often shows an aggressive growth pattern and has been associated with chromosomal imbalances, amplification of oncogenes and epigenetic dysregulation. We present the first genome-wide profiling of JAs (n=14) with a 100K single nucleotide polymorphism (SNP) microarray. Among the 30 novel JA-specific amplifications detected on autosomal chromosomes with this technique, the genes encoding the cancer-testis antigen BORIS (brother of the regulator of imprinted sites) and the developmental regulator protein TSHZ1 (teashirt zinc finger homeobox 1) were selected for further analysis. Gains for both *BORIS* (20q13.3) and *TSHZ1* (18q22.3) were confirmed by quantitative genomic PCR. Furthermore, quantitative RT-PCR revealed a significant up-regulation of *BORIS* ($p<0.001$) and *TSHZ1* transcripts ($p<0.05$) for JAs compared to nasal mucosa. Following detection of BORIS and TSHZ1 proteins in Western blots of JAs, subcellular localization was determined for both proteins in immunostaining of JA cryosections. In conclusion, genomic copy number profiling using an SNP microarray has been proven to be a suitable and reliable tool for identifying novel disease-related genes in JAs and newly implicates BORIS and TSHZ1 overexpression in the pathogenesis of JAs. Detection of BORIS in JAs is described with special regard to tumor proliferation and epigenetic dysregulation, and the finding of *TSHZ1* amplifications is discussed with special respect to the hypothesis of JAs as malformations of the first branchial arch artery.

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Introduction

Juvenile angiofibroma (JA) is an uncommon fibrovascular tumor arising almost exclusively in adolescent males close to the sphenopalatine foramen in the posterior nasal cavity. Blood supply of this hypervascular tumor is usually derived from the maxillary/sphenopalatine artery, however, the internal carotid artery must also be considered as a potential tumor feeder (1). Tumor architecture is characterized by numerous irregularly configured vessels set in a fibrous stroma. Even though this tumor is regarded to be histologically benign, an aggressive growth pattern is frequently observed (2).

Localization and blood supply have led to the hypothesis of JAs as vascular malformations (VM) arising from remnants of the first branchial arch artery (3,4). Recently, this hypothesis has been supported on the molecular level by detection of laminin α2, which is a marker of early angiogenesis, in the perivascular extracellular matrix of JAs (5). However, there have been no detailed investigations into the expression of regulatory factors associated with the development of derivates from the first branchial arch in juvenile angiofibromas. In this context, TSHZ1 (teashirt zinc finger homeobox 1), whose ortholog has been detected in neural crest-derived mesenchymal cells of the first and second branchial arches in mice (6,7), would be an attractive candidate to shed light on the tumor origin of JAs.

The increased incidence of JAs in patients with familial adenomatous polyposis coli (APC) prompted the first genetic studies on JAs (8) and suggested an involvement of the Wnt pathway. APC is part of the β-catenin destruction complex in the Wnt signaling cascade. Activation of the

Wnt pathway inhibits the APC protein, thus increasing the cytoplasmatic pool of β-catenin, which is then translocated to the nucleus, where it acts as a transcriptional co-activator (9). Although alterations of the *APC* gene were not found in JAs (8), activating β-catenin gene (*CTNNB1*) mutations were frequently detected (10). Translocation of β-catenin into the cell nucleus together with androgen receptors (AR) and subsequent activation of AR dependent genes has been suggested to contribute to the preponderance of the tumor in male adolescents (11).

Following these initial mutation studies, comparative genomic hybridization (CGH) and fluorescence *in situ* hybridization (FISH) have been established as valuable genetic tools in JA research (12). In our previous study, numerous chromosomal imbalances were detected in JAs by CGH and FISH (11,13,14). In this context, the observed amplification of *AURKA* (aurora kinase A) and *MDM2* (Mdm2 p53 binding protein homolog) genes is of special interest, as both are discussed to play a role in chromosomal instability in cancers (15). Additionally, FISH revealed gains of the *MYC* oncogene in advanced tumor stages (16). Besides these genetic alterations associated with cell proliferation, loss of imprinting (LOI) has also been observed in JAs (17) indicating a role of epigenetic dysregulation in this rare fibrovascular tumor.

Epigenetic dysregulation is a common feature in human cancers. In recent years, the transcription factor CTCF (CCCTC-binding factor) and its paralog BORIS (brother of the regulator of imprinted sites) have been discussed as key players in this context (18). CTCF is a ubiquitously expressed, highly conserved multivalent transcription factor. Apart from controling cell proliferation-associated genes, this methylation-sensitive DNA binding-protein is also involved in epigenetic regulation (19). Normally, the expression of CTCF and BORIS is mutually exclusive with BORIS almost completely restricted to spermatocytes in the male testis where it controls imprinting in the male germ line development (20). However, an aberrant expression of BORIS has been detected in various cancer cell lines and native tumors, where it is supposed to interfere with the normal regulatory functions of CTCF (18). Therefore, BORIS is regarded as a cancer-testis antigen (CTA) (21).

To the best of our knowledge, genetic alterations of either *BORIS* or *TSHZ1* have not been analyzed in tumors using single nucleotide polymorphism (SNP) oligonucleotide microarrays to date. Due to the increased resolution of SNPs compared to CGH, this technique of molecular karyotyping has gained growing importance in the genome-wide analysis of genetic alterations in recent years including screening for tumor-susceptibility genes (22).

We report the first application of array-CGH in JAs revealing gains in *TSHZ1* and *BORIS* genes as a common finding. These observations were confirmed by increased levels of both *TSHZ1* and *BORIS* mRNA in JA tissue compared to nasal mucosa and detection of TSHZ1 and BORIS proteins in JAs. These novel results are discussed in the context of JA tumor origin, tumor proliferation and epigenetic dysregulation.

Materials and methods

Tissue and blood samples. Two different sets of tissue and blood samples were used in this study. Set I comprised 14 JA tissue specimens, peripheral blood from 2 of the 14 JA patients and from 6 healthy controls. These samples were objected to molecular karyotyping using a 100K SNP array and genomic PCR analysis. Due to limited amounts of tumor tissue from set I, a different set of samples (set II) including 15 JAs and 11 nasal mucosa (NM) specimens was used for comparative BORIS and TSHZ1 mRNA/protein expression studies.

All JAs analyzed in this study were-snap frozen immediately after tumor removal. Tumor stages ranged from stage II to stage IIIb according to Andrews (23). Beside the characteristic clinical findings, diagnosis was confirmed by detailed histological and immunohistological studies, as previously reported (16). Nasal mucosa used as control tissue was derived from inferior turbinate surgery for treatment of nasal obstruction in otherwise healthy individuals. For transcript analysis, the superficial epithelial layer of the NM was removed in order to obtain a homogeneous stromal tissue sample comparable to the texture of JAs. All patients were informed and gave their written consent.

100K SNP array. DNA (500 ng) from each of the 14 JA tumor specimens and from two blood samples of affected patients (set I) was processed and analyzed with the Affymetrix GeneChip® Human Mapping 100K array (Affymetrix, Santa Clara, CA, USA), as previously described (24). Hybridization and data acquisition were performed by the Gene Mapping Center, Max-Delbrück-Center for Molecular Medicine in Berlin-Buch (Germany). Call rates exceeded 98% in all analyzed samples. Raw data were processed with the Affymetrix copy number analysis tool (CNAT) using 0.5 Mb sliding windows for the genomic smoothening algorithm (GSA). The CNAT copy number output was exported to a table, which was then filtered for clusters of SNPs fulfilling certain criteria using the Copy Number Variation Finder (CNVF) (24). Using this software tool, copy number data were filtered for blocks fulfilling the following conditions: GSA p-values for duplications >+2.5 (at least 3 SNPs with a value >+5) and for deletions <-2.5 (at least 3 SNPs with a value <-5). The Y-chromosome was not covered by the used SNP array.

Quantitative PCR

a) Genomic PCR. Sufficient DNA for quantitative (q) genomic PCR was available from 12 JAs and 6 blood samples from healthy controls (set I). Before performing TaqMan® gene expression assays (Applied Biosystems, Darmstadt, Germany) and data analysis as previously described (25), optimal concentrations of primers, probes and templates were determined on separate multiplexed optimizing pre-runs. Primer Express 2.0 software was used to design the TaqMan primers and probes from GenBank sequences no. NM_005786 (*TSHZ1*) and no. NM_080618 (*BORIS*). Primer sequences were as follows: *TSHZ1* forward, GCAAGAGACCGCGGAACAT; *TSHZ1* reverse, GCTCAGGCCAGGCCTAGTG; *TSHZ1* probe, CACTAAACGTCGTCGAGC; *BORIS* forward, ACGAGTTCTCCACTGGTGACAA, *BORIS* reverse, GGAACCAGGCCCTACAAGTGT and *BORIS* probe, TGCCATGTTGCAGTCG). For determination of gene copy numbers, the ΔΔCt method based on threshold cycle numbers (Ct) was employed. Our previous study showed that a ΔΔCt ratio >1.3 identified 100% of gene amplifications in blood

samples from patients with a *PMP22* duplication (26), whereas ΔΔCt values from 0.8 to 1.2 confirmed a diploid (normal) genotype. Due to the contamination of JAs with normal DNA from non-tumor cells, the exact ΔΔCt value for gains is uncertain. Considering the heterogeneous cellular composition of JAs with at least 30% inflammatory cells (27), a modified ΔΔCt ratio >1.2 for a sample was judged as an indicator for gene duplications in this study.

b) Reverse transcriptase (RT) PCR. Total RNA was isolated from the 15 JAs and 11 nasal mucosa samples from set II using illustra™ RNAspin (GE-Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions, including genomic DNA digestion. Total RNA (1 μg), determined by photometrical analysis, was used for reverse transcription (QuantiTect RT-Kit, Qiagen, Hilden, Germany), obtaining 20 μl of final volume.

For all qRT-PCR reactions, the qPCR-Core-Kit for SYBR-Green I No Rox (Eurogentec, Cologne, Germany) and StepOnePlus Real-Time-PCR devices (Applied Biosystems, Darmstadt, Germany) were used. Standard curves for quantifying copy numbers were generated using respective products from former test PCRs, which were extracted from agarose gel after electrophoresis. The purified template cDNA allowed serial dilutions for different copy numbers (10^1 to 10^7). For absolute quantification, the copy number of target genes was extrapolated from the standard curve equation.

For normalization, GeNorm software algorithm (28) selected *HPRT1* (hypoxanthine phosphoribosyltransferase 1), *TUBA1A* (tubulin α 1a) and *RPS18* (ribosomal protein S18) as the most suitable out of four housekeeping genes (*HPRT1, TUBA1, RPS18, GAPDH*). Primer pairs for qRT-PCR were *BORIS* forward, GAGCGGGACCATGAAAATACATA and *BORIS* reverse, ATATGCACACGTAGGTCGCTTTT (114 bp, 62˚C annealing temperature); *TSHZ1* forward, CGCAGTCAAACTGCACCTTA and *TSHZ1* reverse, CTCG ACGACGTTTAGTGCAA (134 bp, 61.5˚C); *RPS18* forward, AGTTCCAGCATATTTTGCGAGTA and *RPS18* reverse, TTTCCATCCTTTACATCCTTCTG (259 bp, 61.4˚C); *HPRT1* forward, GACCAGTCAACAGGGGACAT and *HPRT1* reverse, CCTGACCAAGGAAAGCAAAG (132 bp, 59˚C); *TUBA1* forward, GATTATGGCAAGAAGTCCAAGC and *TUBA1* reverse, TACCATGAAGGCACAATCAGAG (135 bp, 59˚C).

Statistical analysis. Statistical calculations were carried out using the Kruskal-Wallis test and Dunn's multiple comparison test. Differences with a $p<0.05$ were considered as significant.

Western blot analysis. Of each tissue sample, 25-40 mg were homogenized (T25 Basic, IKA Labortechnik, Staufen, Germany) in 1 ml lysis buffer (50 mM Tris pH 7.4, 300 mM NaCl, 10 mM EDTA, 1% NP40, 0.1% Triton X-100, 0.1% SDS, protease inhibitor cocktail Complete® (Roche, Mannheim, Germany). Samples were incubated at 4˚C for 2 h and centrifuged with 16,000 g for 1 h. Protein concentration of the supernatant was analyzed using BCA assays (Pierce Biotechnology, Rockford, USA) and aliquots were stored until usage at -80˚C.

After denaturation (3 min at 90˚C with SDS loading buffer including mercaptoethanol), 50 μg of lysate was applied to each lane, separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred to nitrocellulose membranes (Protran-BA-83, Schleicher & Schuell, Dassel, Germany). Ponceau S staining served as additional loading control. For protein detection, primary antibodies against BORIS (1:2000, rabbit polyclonal ab18337, Abcam Inc., UK, Cambridge) and TSHZ1 (1:200, rabbit polyclonal M5943, Sigma, Taufkirchen, Germany) diluted in RotiBlock (Carl Roth, Karlsruhe, Germany) were used. HRP-conjugated F(ab')2 goat anti-rabbit IgG (H+L) secondary antibodies (KPL, Gaithersburg, USA), diluted 1:5000 in RotiBlock, were applied for 60 min, followed by peroxidase substrate Supersignal West Dura (Thermo Scientific, Bonn, Germany) for 5 min. Finally, signals were detected by the LumiImager-F1 (Roche, Mannheim, Germany).

Immunofluorescence staining. Immunofluorescence staining of freshly prepared 5 μm cryosections was performed using an affinity-purified rabbit polyclonal TSHZ1 antibody (1:200, M5943, Sigma, Taufkirchen, Germany) and an affinity-purified rabbit polyclonal BORIS antibody (1:200, ab18337, Abcam Inc., Cambridge, UK). To differentiate expression of BORIS and TSHZ1 in endothelial and stromal mesenchymal cells, co-localization studies with the mouse anti-CD31 antibody as an endothelial marker (1:40, JC-70A, Dako, Germany) and the mouse anti-prolyl-hydroxylase β antibody as a marker of mesenchymal stromal cells (1:400, clone 3-2B12, Acris, Hiddenhausen, Germany) were performed, respectively. DAPI (Vector Laboratories, Burlingame, CA, USA) was used for nuclear counterstaining. Sections were analyzed and photographed with the APOTOME technique (Zeiss, Jena, Germany).

Results

Detection of BORIS and TSHZ1 gains in JAs. In order to identify small genome aberrations specific for juvenile angiofibromas, genome-wide copy number profiling using a 100K SNP array with a resolution down to 200 kb was performed on 14 JA samples and peripheral blood from two of the 14 JA patients (set I). Compared to the blood samples, where only copy number variations common in the general population were detected, a large number of unusual aberrations was found in each of the 14 investigated tumors (data not shown). By using the strict evaluation algorithm described above, 30 duplications on autosomal chromosomes were identified. Of those, four were detected in 7/14 and two were found in 8/14 tumor samples (Table I, Fig. 1). Among these interesting candidate genes for JA-specific gains, two with possible implications for JA biology were selected for further analysis: *BORIS* (20q13.3), which was amplified in 8/14 JAs, was chosen because its gene product is known as a cancer-testis antigen involved in cell proliferation and epigenetic dysregulation of cancers (18). Second, *TSHZ1* (18q22.3), which showed gains in 7/14 tumor specimens, emerged as an attractive candidate gene with respect to our hypothesis of JAs as vascular malformations of the first branchial arch artery (4,7,29).

Quantitative genomic PCR from 12 of the 14 JA tumors and six peripheral blood samples from healthy controls (set I)

Table I. JA-specific chromosomal alterations detected by the 100K SNP DNA microarray.

Location	rs-number	SNP	Location in bp	Affected tumors
1p33	rs10489770-rs1002378	SNP_A-1672946-SNP_A-1699173	46580180-47317900	Gains in 3 out of 14
1p31.1	rs10493484-rs2125775	SNP_A-1653047-SNP_A-1688718	71474040-71474050	Gains in 5 out of 14
1q23.3	rs10494435-rs10494442	SNP_A-1702658-SNP_A-1650800	163442185-163521850	Gains in 3 out of 14
1q44	rs2365082-rs2362932	SNP_A-1732549-SNP_A-1649226	243398800-243980910	Gains in 3 out of 14
2p25.1	rs7570654-rs10495548	SNP_A-1737596-SNP_A-1661317	6874422-7054890	Gains in 4 out of 14
2q22.3	-rs274844	SNP_A-1710906-SNP_A-1714655	-145634550	Gains in 3 out of 14
3p22.1	rs2371319-rs422623	SNP_A-1699128-SNP_A-1696672	40701945-41098520	Gains in 7 out of 14
3p11.1	rs1483489-rs3946805	SNP_A-1743831-SNP_A-1655924	88963955-89399070	Gains in 5 out of 14
3q26.32	rs9290637-rs4076914	SNP_A-1755631-SNP_A-1657013	179374400-180154385	Gains in 3 out of 14
7p12.3	rs2768419-rs10240495	SNP_A-1755341-SNP_A-1665150	49502965-49791960	Gains in 8 out of 14
8q11.21	rs1372062-rs341817	SNP_A-1686924-SNP_A-1664173	50028400-50186155	Gains in 3 out of 14
8q22.1	rs6991577-rs7828725	SNP_A-1654545-SNP_A-1676299	93948600-94208095	Gains in 3 out of 14
8q22.1	rs10504974-rs1872011	SNP_A-1715057-SNP_A-1657906	98591510-98888600	Gains in 3 out of 14
8q24.13	rs4128469-rs723231	SNP_A-1753969-SNP_A-1673612	125548650-126018235	Gains in 4 out of 14
9p21.3	rs1329036-rs10491795	SNP_A-1653471-SNP_A-1685579	23819910-24103907	Gains in 4 out of 14
9q31.2	rs4978698-rs4259479	SNP_A-1741597-SNP_A-1652845	110349970-110564480	Gains in 3 out of 14
9q33.1	rs7032395-rs10513300	SNP_A-1697364-SNP_A-1701826	118867780-119170030	Gains in 3 out of 14
9q34.13	rs10512414-rs4128956	SNP_A-1717146-SNP_A-1721113	133325225-133808330	Gains in 4 out of 14
10q22.1	rs1417909-rs7918636	SNP_A-1723364-SNP_A-1645168	72592370-72885535	Gains in 3 out of 14
10q26.13	-rs2292623	SNP_A-1672547-SNP_A-1679262		Gains in 7 out of 14
11q25	rs1793821-rs1355982	SNP_A-1754058-SNP_A-1736383	130525080-130853300	Gains in 7 out of 14
12q21.33	rs1504143-rs10492233	SNP_A-1657346-SNP_A-1713503	89744900-89938450	Gains in 5 out of 14
12q23.2	rs825041-rs10507135	SNP_A-1665482-SNP_A-1703736	100485005-100853956	Gains in 3 out of 14
13q.34	rs496916-rs718886	SNP_A-1673670-SNP_A-1666004	109649015-110044380	Gains in 4 out of 14
14q32.2-q32.31	rs736319-rs2152369	SNP_A-1670897-SNP_A-1716060	99959035-100696370	Gains in 3 out of 14
15q21.1	rs2460620-rs8039174	SNP_A-1662257-SNP_A-1727759	44085750-44485440	Gains in 4 out of 14
16q23.1	rs1364095-rs1423815	SNP_A-1693240-SNP_A-1708947	78123651-78194350	Gains in 3 out of 14
18q12.2	rs10502699-rs10502705	SNP_A-1704978-SNP_A-1699983	34337732-34477370	Gains in 4 out of 14
18q22.3	**rs6566067-rs9319680**	**SNP_A-1746537-SNP_A-1647465**	**71104940-71260335**	**Gains in 7 out of 14**
20q13.3	**rs911901-rs326832**	**SNP_A-1758638-SNP_A-1698862**	**55385594-55952451**	**Gains in 8 out of 14**

Data associated with the chromosomal localization of *TSHZ1* (18q22.3) and *BORIS* (20q13.3) is bold-typed.

was used for validation of the results obtained with the 100K SNP assay. To rule out a common SNP polymorphism for the selected genes, control DNA from blood of healthy donors (n=6) was studied first (Fig. 2, control 1-6). Here, qPCR of genomic DNA for both candidate genes produced ΔΔCt values ranging from 0.97 to 1.08, indicating a diploid genotype. A ΔΔCt value >1.2 confirmed *BORIS* gene duplication in five of the six tumors which had shown *BORIS* gains in the 100K SNP array (Fig. 2A, cases 7-12). On the contrary, ΔΔCt values were <1.2 for 6/6 JA specimens lacking an amplification of *BORIS* in the microarray (Fig. 2A, cases 1-6). Duplication of the *TSHZ1* gene was confirmed in 6/6 JAs by a ΔΔCt value >1.2 in qPCR (Fig. 2B, cases 7-12). Each of the six tumors without a detectable gain in *TSHZ1* in the 100K SNP array showed a ΔΔCt value <1.2, which is in agreement with a diploid genotype (Fig. 2B, cases 1-6).

Expression of BORIS and TSHZ1 gene products in JAs. As the amount of tumor tissue from the 14 JA samples of set I was not sufficient for further investigations, a different set of 15 JAs and 11 nasal mucosa specimens (set II) was used for expression analysis of BORIS and TSHZ1 on the mRNA and protein level.

For both genes, mRNA copy numbers were significantly higher in JAs compared to the subepithelial stroma of nasal mucosa (*BORIS*, $p<0.001$; *TSHZ1,* $p<0.05$) (Fig. 3). In order to investigate whether the up-regulation of *BORIS* and *TSHZ1* transcripts in JAs was reflected on the translational level, protein samples available from eight JAs and six nasal mucosa specimens (set II) were objected to Western blotting. A signal of about 83 kD was detected for BORIS protein in 8/8 JAs and in 3/6 inferior turbinates (Fig. 4). For TSHZ1, a band at approximately 115 kD was observed for 8/8 JAs and 6/6 nasal mucosa samples (Fig. 4). On visual inspection, TSZH1 signal intensity was lower for all NM compared to JA specimens.

In order to determine the localization of BORIS and TSHZ1 proteins in juvenile angiofibromas, immunofluorescence analyses of JA cryosections were performed. A dot-like

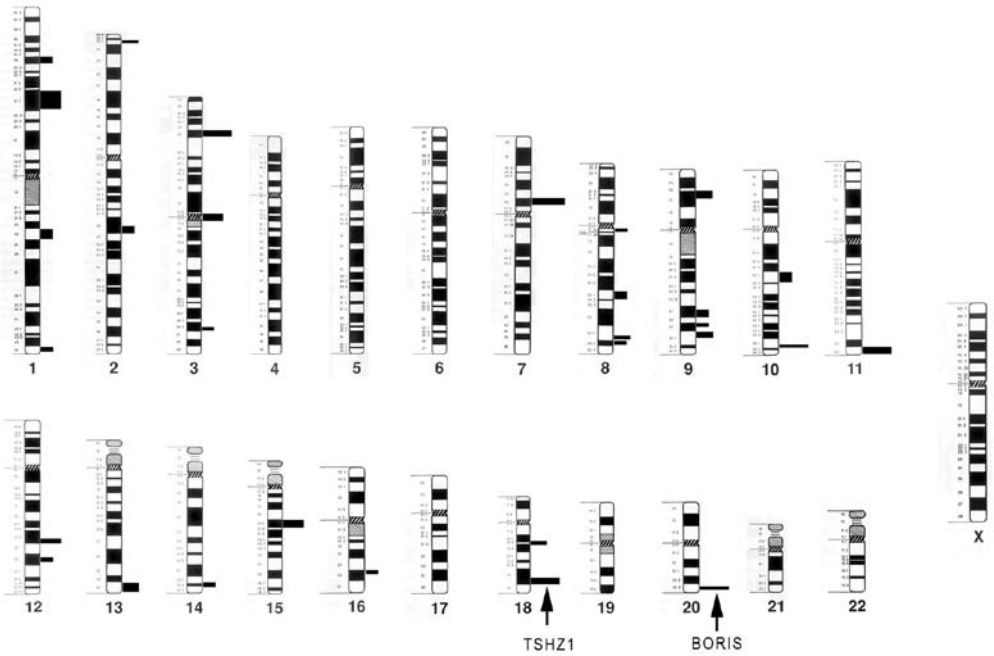

Figure 1. Ideogram of the duplications detected in 14 JAs using a 100K SNP array with genomic smoothening algorithm (GSA)-p-values >+2.5 for duplications (at least 3 SNPs >+5) and <-2.5 for deletions (at least 3 SNPs <-5). The widths of the bars indicate the number of angiofibromas in which previously unknown gains were detected.

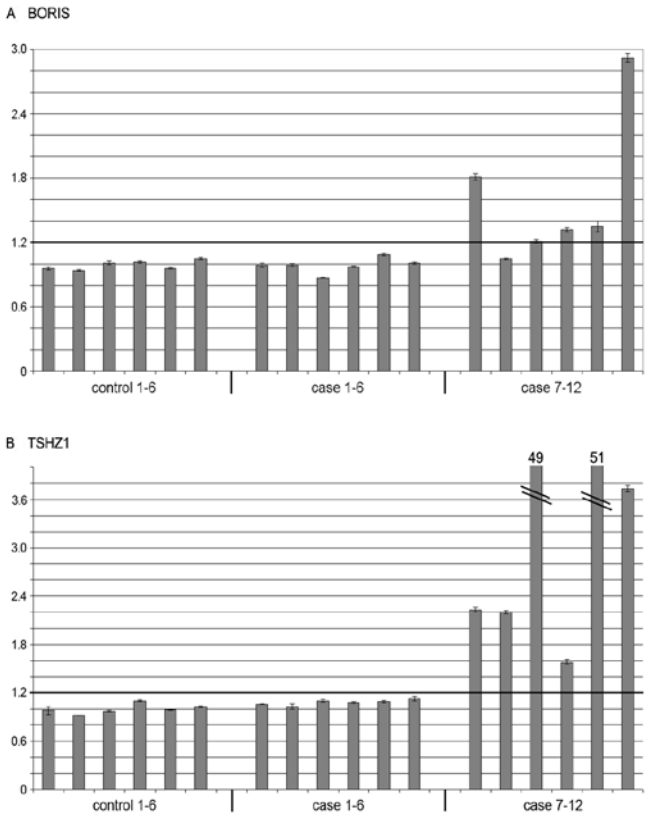

Figure 2. Results of genomic qPCR for *BORIS* (A) and *TSHZ1* (B). ΔΔCt values from 0.8 to 1.2 (y-axis) were judged as diploid (normal) genotype, whereas a ΔΔCt value >1.2 was interpreted as an amplification. Blood samples from 6 healthy individuals (controls 1-6) displayed ΔΔCt values <1.2 for *BORIS* and *TSHZ1*. (A) ΔΔCt values were <1.2 for 6/6 JAs without gains for *BORIS* in the 100K SNP array (cases 1-6), whereas 5/6 JAs with *BORIS* duplication in the microarray showed ΔΔCt values >1.2 (cases 7-12). (B) ΔΔCt values <1.2 were detected for 6/6JAs without gains for *TSZH1* in the 100K SNP array (cases 1-6). 6/6 JAs with *TSHZ1* amplifications in the microarray displayed ΔΔCt values >1.2 (cases 7-12).

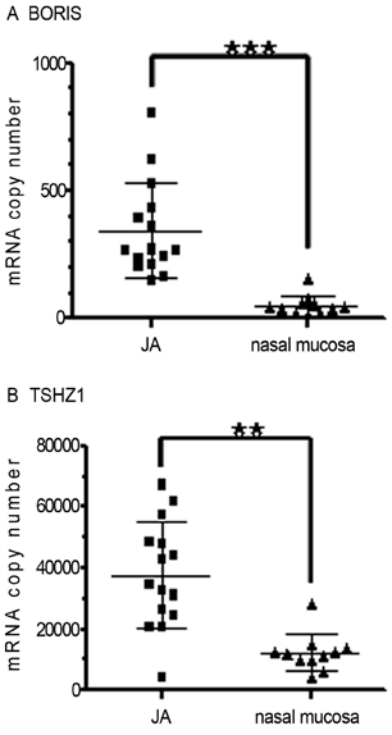

Figure 3. *BORIS* (A) and *TSHZ1* (B) mRNA expression in JAs compared to nasal mucosa. qRT PCR revealed a significant up-regulation of *BORIS* (***p<0.001) and *TSHZ1* transcripts (**p<0.05) in JAs compared to the subepithelial stroma of nasal mucosa from control persons.

predominantly nuclear expression was detected for BORIS (Fig. 5A, red, arrow). Co-staining with the endothelial marker CD31 (green) revealed BORIS protein in the cell nuclei of endothelial (EC) and perivascular cells surrounding

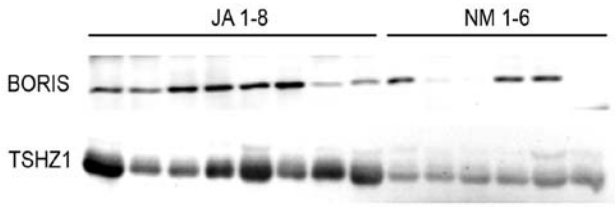

Figure 4. Western blot analysis of BORIS and TSHZ1 protein expression in 8 JAs (JA 1-8) and 6 specimens of nasal mucosa (NM 1-6). A signal for BORIS was detected at 83 kD for 8/8 JAs and 3/6 NM samples. TSHZ1 protein was identified at 115 kD in 8/8 JAs, a faint signal for TSHZ1 was visible in 6/6 NM specimens.

blood vessels (V) and in mesenchymal cells (MC) (Fig. 5A). Visualization of the mesenchymal stromal cell marker protein prolyl-hydoxylase β (green) identified BORIS (red, arrow) also in the cytoplasm of JA stromal cells (Fig. 5B, yellow signal). TSHZ1 showed a punctuated, mainly cytoplasmatic staining pattern (Fig. 5C and D; red, arrow). Most prominent staining for TSHZ1 around JA blood vessels (V) was seen in perivascular cells (Fig. 5C, PC). Dot-like TSHZ1 signals were also detected in endothelial cells (Fig. 5D, EC), and co-localization (yellow) of TSHZ1 (red) with prolyl-hydoxylase β (green) confirmed expression of TSHZ1 in the cytoplasm of stromal cells (Fig. 5D, MC).

Discussion

We present the first application of an array-based molecular karyotyping for genome-wide copy-number profiling in JAs. Gains for *BORIS* and *TSHZ1* detected by the microarray were confirmed using quantitative genomic PCR. Up-regulation for both *BORIS* and *TSHZ1* transcripts was observed in qRT-PCR analysis, and both proteins were detected by Western blotting and immunostaining of JA cryosections. Their possible role in JA tumor origin, proliferation and epigenetic dysregulation is discussed below.

Expression of the cancer-testis antigen BORIS in JAs. BORIS maps to the so-called cancer amplification region at chromosome 20q13. Gains in this region have been identified in many human cancers leading to the suggestion that it harbors a major oncogene or dominant immortalizing genes promoting genetic instability (18). Detection of chromosomal gains on this 'tumor hot spot' in 8/14 JAs in the present study (Table I, Fig. 1) fits with the observation that JAs often show an aggressive tumor behavior like cancers, although they are histologically classified as benign (2). The gene of the BORIS paralog CTCF is located on the long arm of chromosome 16 (16q22). Loss of heterozygosity (LOH) in this region has been described for a number of cancers (18). In addition, our previous CGH studies revealed chromosomal losses for 16q in 6/27 JAs (15). Taken

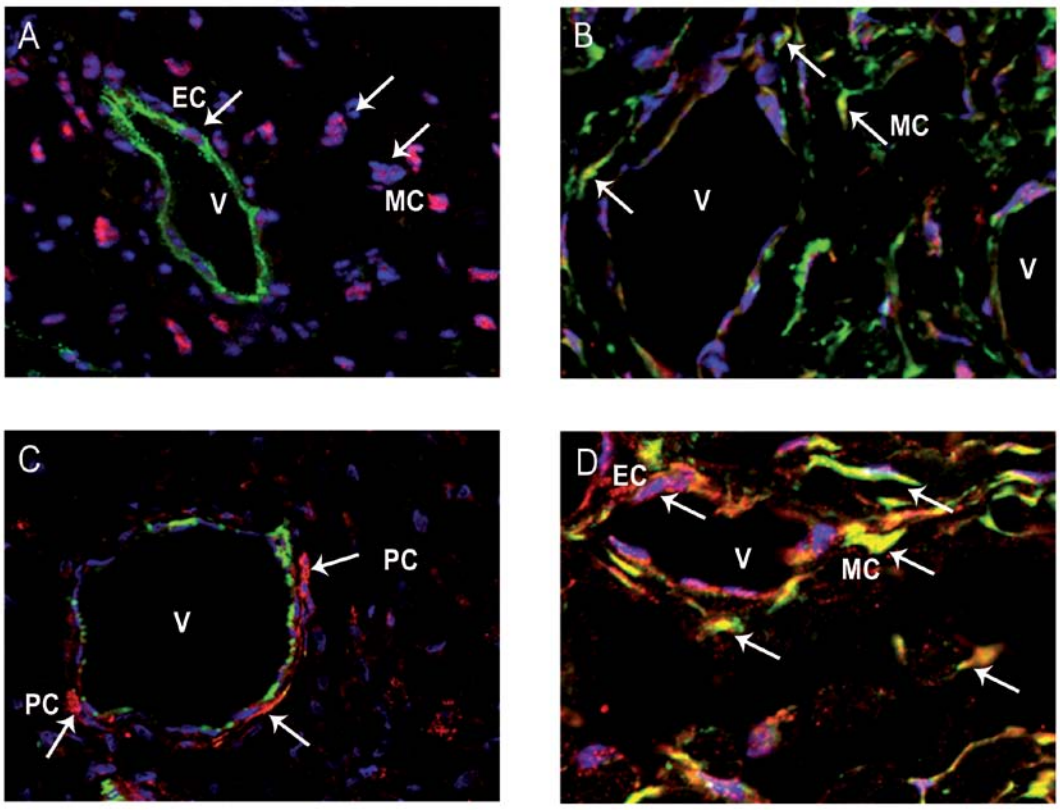

Figure 5. Immunostaining of BORIS and TSHZ1 protein in cryosections of JAs. Cell nuclei were counterstained with DAPI (blue). (A) Endothelial cells were stained with CD31 (green). BORIS (red, arrow) was detected in the cell nuclei of endothelial (EC, green), perivascular and mesenchymal cells (MC). The lumen of the vessel is indicated by 'V'. (B) An antibody against prolyl-hydroxylase β (green) was used as a cytoplasmic marker for mesenchymal stroma cells. BORIS protein (red, arrow) was co-localized with prolyl-hydoxylase β (green) in the cytoplasm (yellow signal) of mesenchymal cells (MC). (C) Co-staining of JA vessels with an antibody against TSHZ1 (red, arrow) and the endothelial marker CD31 (green) visualized TSHZ1 protein in perivascular cells (PC). (D) TSHZ1 (red, arrow) was detected in the cell nuclei of endothelial cells (EC) and co-localizes (yellow signal) with prolyl-hydoxylase β (green) in the cytoplasm of mesenchymal cells (MC).

together, the genetic alterations on chromosome 20q and 16q observed in JAs in our present and previous studies suggest a role of CTCF/BORIS competition in the pathogenesis of this unique tumor.

BORIS belongs to the group of cancer-testis antigens (CTA), which are normally almost exclusively found in testis, but are abnormally activated in cancer cells (18). As testis is an immune-privileged organ, CTAs may be considered as non-self antigens capable of eliciting strong anti-cancer immunity. Due to their immunogenicity and tumor-restricted expression, several CTAs have been used in recent years to develop targeted anti-tumor vaccines (21). BORIS is not only a cancer-testis antigen itself, but has also been associated with derepression of other CTAs including NY-ESO-1 and MAGE-A1 (30,31). Therefore, BORIS has become an attractive candidate antigen for tumor vaccination. Recent studies showed that a DNA vaccination against BORIS inhibited tumor growth and prolonged survival in a mouse model of mammary adenocarcinoma (32,33).

In the present study, a significant up-regulation of *BORIS* transcripts was observed in JAs compared to the subepithelial stroma of nasal mucosa (Fig. 3A). Low-level expression of *BORIS* mRNA in nasal mucosa from healthy individuals is in line with the previous detection of small amounts of *BORIS* mRNA in pancreas, prostate, thymus and kidney. Consequently, *BORIS* is regarded as a 'differentially expressed CT gene', whose transcripts can be detected in normal tissues apart from testis, albeit at a lower level (21). Observation of BORIS protein in some control tissue samples in two studies investigating BORIS expression in breast cancer (34,35) fits with this concept. Identification of BORIS protein in 3/6 nasal mucosa samples compared to 8/8 JAs in the present study (Fig. 4) is in line with the notion that BORIS can indeed be found in non-tumor tissues. Due to the heterogeneity of native nasal mucosa with a high proportion of glands in the epithelium, a quantitative interpretation of protein signals in Western blots comparing NM and JA is not advisable. Furthermore, it has to be considered that only a small number of JAs (n=8) was available for this study. As JA is such a rare tumor, multi-center studies would be necessary in the future in order to obtain the exact relation of BORIS protein expression in JA versus nasal mucosa samples. In this context, a potential impact of BORIS expression in normal tissue on the immunogenic potential of this CTA needs further evaluation (21). Nevertheless, the option of designing an anti-JA tumor vaccine in the future is of high clinical interest as currently no effective drug treatment is available for this fibrovascular tumor. Anti-tumor vaccination would offer an attractive opportunity to induce tumor shrinkage of advanced JAs prior to operation.

Potential impact of BORIS on JA cell proliferation, hormone signaling and epigenetic dysregulation. The ubiquitously expressed transcription factor CTCF regulates numerous genes associated with cell cycle regulation and cell proliferation. Aberrant expression of BORIS is suggested to compete with CTCF in regulation of its target genes (18). Studies from immature B cells have shown that CTCF controls both *MYC* and *MDM2* oncogenes (36). Gains for both genes have been identified for JAs in our previous studies (15,16). Detection of BORIS in JAs in the present study raises the question whether it activates *MYC* and *MDM2* in this fibrovascular tumor. Recently, Nguyen *et al* (37) observed binding of BORIS protein to the *MYC* promoter and down-regulation of *MYC* transcripts induced by small hairpin (sh) RNA knockdown of BORIS in colon carcinoma cells suggesting that BORIS enhances *MYC* expression in cancer cells. Further investigations will be necessary to determine whether the molecular mechanisms of BORIS/MYC interaction described above can be verified for JAs and whether BORIS interferes with CTCF-associated regulation of MDM2.

Beside its impact on cell-proliferation associated genes, BORIS has been reported to interfere with hormone receptors in human cancers. A recent study did not only identify BORIS protein in 70% of the analyzed breast cancer specimens, but also correlated high levels of BORIS with high levels of estrogen receptor (ER) and progesterone receptor (PR) proteins. Moreover, BORIS was shown to activate the promotors of both ER and PR genes in reporter assays (34). Concerning JAs, expression and function of sex steroid receptors are still controversial issues (38-40). A recent study detected P450 aromatase, which is able to convert androgens into estrogens, in 5/5 analyzed JAs (41). Future investigations into a possible role of BORIS in hormone-dependent gene regulation in JAs may shed light on the interplay of estrogen, progesteron and androgen in this tumor, which almost exclusively afflicts adolescent males.

CTCF interacts with chromatin insulators/boundaries, which partition the genome into distinct functional domains preventing long-distance communication of *cis* regulatory elements, e.g. promotors, with enhancers/silencers (19). This role of CTCF in epigenetic regulation has extensively been studied for the *H19/IGF2* imprinting control region (ICR). IGFs (insulin like growth factors) have been suggested to be involved in tumorigenesis by promoting cell growth and division (42). CTCF is only able to bind to the unmethylated ICR on the maternal allele, thus blocking the communication between the *IGF2* promotor and a downstream enhancer resulting in silencing of *IGF2*. Methylation of the maternal *H19/IGF2* ICR in cancer cells abrogates binding of CTCF leading to derepression of *IGF2* gene expression (19). In contrast to its paralog CTCF, BORIS is a methylation-independent DNA-binding protein that preferentially binds to the methylated *H19/IGF2* ICR (43). Due to these observations, BORIS is supposed to play a central role in epigenetic dysregulation including loss of imprinting (LOI), which is a common feature in many cancers. LOI for the *IGF2* gene (17) and up-regulation of *IGF2* mRNA (44) have also been observed in JAs previously. Further investigations will be necessary to elucidate whether BORIS is involved in epigenetic dysregulation of *H19/IGF2* and other genes in JAs.

Expression of TSHZ1 in JAs. In mice, *Tshz1* gene products have been located to neural crest-derived mesenchymal cells of the first and second branchial arches (6) and have been associated with development of the soft palate and the middle ear (7). Here, we are the first to describe amplification of the human ortholog *TSHZ1* in JAs (Figs. 1 and 2) resulting in an up-regulation of *TSHZ1* transcripts compared to nasal mucosa (Fig. 3B). Western blotting revealed TSHZ1 protein expression in 8/8 JAs, while only a faint protein signal was detected in 6/6 nasal mucosa specimens (Fig. 4). The pivotal role of Tshz1

in the development of the soft palate and middle ear (7) offers an explanation for low-level expression of TSHZ1 in nasal mucosa, which forms the epithelial surface of both the soft palate and the middle ear. Up-regulation of *TSHZ1* mRNA (Fig. 3B) and localization of TSHZ1 protein in perivascular and mesenchymal cells in JAs (Fig. 5C and D) are interesting findings concerning the origin of JAs: anatomical studies on blood supply (4) of JAs and the vascular architecture of this fibrovascular tumor (5) have given rise to the hypothesis of JAs evolving from remnants of the first branchial arch artery. Identification of the developmental regulatory factor TSHZ1, whose mouse ortholog is found in mesenchymal cells of the first branchial arch, in JAs adds further evidence to this hypothesis on the molecular level.

Possible roles of TSHZ1 in JA tumorigenesis. The *Drosophila* ortholog of *TSHZ1, tsh (teashirt)*, is required for maintenance of the Wg (wingless) pathway, which corresponds to the Wnt signaling cascade in vertebrates (45,46). The Wnt pathway is not only essential for body plan formation during embryonal development (47), but is also involved in tumorigenesis of many cancers (48). Considering previous evidence for a role of Wnt signaling in JAs (12), a comparison between the Wg and Wnt pathways regarding the role of *tsh* and *TSHZ1* may offer new insights into tumor biology of JAs: in *Drosophila*, Tsh binds to and stabilizes the transcription factor Armadillo (Arm), an analog to β-catenin in vertebrates (45,49). Furthermore, a *Xenopus laevis* ortholog of Tsh, XTsh3, has been shown to enhance Wnt signaling by binding to β-catenin and enhancing β-catenin levels in the nucleus (50). In JAs, frequent activating β-catenin mutations have been detected previously (10). These observations raise the question whether TSHZ1 is a binding partner of β-catenin in JAs capable of shaping Wnt signaling in this rare fibrovascular tumor.

Beside their role in embryonal development, gene products of *teashirt* orthologs also have to be considered as cancer antigens. TSHZ1 antigen was identified in a human cDNA expression library derived from human colon cancers and is therefore also known as SDCCAG 33 (serologically defined colon cancer antigen 33) or NY-CO-33 (21). Reactivation of developmental signaling cascades like the Wnt pathway is a common feature in many tumors.

In conclusion, the present study shows that molecular karyotyping using an SNP array is a suitable and reliable approach for detecting genetic alterations in JAs. Using this technique, the products of two regulatory genes were identified as novel tumor markers of JAs: BORIS, which is normally involved in imprinting during male sperm cell development and the colon cancer antigen TSHZ1, whose mouse ortholog is found in derivates of the first branchial arch. Future investigations will be needed to elucidate the underlying molecular mechanisms and to unravel the functional implications of BORIS and TSHZ1 in this unique fibrovascular tumor.

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