Implications of Tropomyosin-related Kinase B (TrkB) in Head and Neck Cancer

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Abstract. *Background: Head and neck squamous cell carcinomas (HNSCC) represent the most common malignancies of the upper aerodigestive tract. Expression of tropomyosin-related kinase B (TrkB) was found to be essential to prevent anoikis in rat intestinal epithelial cells allowing for survival of cells outside the epithelial cell layer and subsequent metastatic spread. The aim of the present study was to examine the potential role of TrkB in HNSCC disease. Materials and Methods: SDS-PAGE and Western blot analysis of TrkB expression were performed under standard conditions. Immunohistochemistry was performed with the Avidin Biotin Complex method, whereas immunocytochemistry was performed by fluorescence staining. Tumor cell invasiveness was measured by an in vitro invasion assay. Results: A variable but consistent expression of TrkB could be observed in HNSCC tissues and cell lines, whereas no significant expression was seen in the healthy control tissue (soft palate). Immunohisto- and cytochemical staining further demonstrated pronounced TrkB expression in HNSCC tumor cells. However, the invasive properties of TrkB-expressing HNSCC cells were not significantly altered after treatment with 10 or 100 ng/ml brain-derived neurotrophic factor, the natural ligand of TrkB. Conclusion: The presented data implicates to a great extent that TrkB plays a significant role in HNSCC disease since it was consistently found to be overexpressed in cells of this tumor entity. We conclude that the role of this receptor tyrosine kinase is rather the prevention of anoikis, as demonstrated earlier, and less the modulation of HNSCC invasiveness, since invasion was not affected after receptor stimulation. Further studies should investigate possible inhibitors of TrkB and their suitability for the treatment of head and neck cancer.*

Early regional lymph node and subsequent distant metastatic spread is a hallmark of tumors such as head and neck squamous cell carcinomas (HNSCC) (1). The prognosis of the patient drops dramatically when lymph node metastases are present at the time of diagnosis. If normal epithelial cells detach from the basement membrane and lose contact with other neighboring cells, they will usually die due to apoptosis. This specific form of apoptosis is called anoikis (2). Douma and co-workers recently identified tropomyosin-related kinase B (TrkB), a receptor tyrosine kinase, as essential for anoikis-resistance of rat intestinal epithelial cells (3). TrkB was first identified in mouse neural development and found to be expressed in most structures of the central and peripheral neural system (4-6). Douma *et al.* demonstrated that normal rat intestinal epithelial cells became resistant to anoikis leading to extensive metastatic spread after transfection and expression of TrkB and even more so after co-expression of its specific ligand brain-derived neurotrophic factor (BDNF) (3). Therefore, it is of great interest to investigate the role of TrkB in tumor pathogenesis. Since its first description in 1989 (4) and its implication in tumor cell survival and metastatic spread (3), there have been several studies reporting a possible role of TrkB not only in diverse tumor entities such as neuroblastoma, prostate cancer, basal cell and cutaneous squamous cell carcinomas (7, 8), but also in myelomas in which the receptor promotes tumor cell survival (9). However, little is known about the potential role of TrkB in HNSCC.

The goal of our investigations, therefore, was to assess the possible significance of TrkB expression in HNSCC by evaluating TrkB expression and changes in the invasiveness of TrkB-positive tumor cells after TrkB-stimulation with BDNF.

Abbreviations: BDNF: brain-derived neurotrophic factor; HNSCC: head and neck squamous cell carcinoma; MDCK-C7: Madin-Darby canine kidney cells clone C7; TEER: transepithelial electrical resistance; TrkB: tropomyosin-related kinase B.

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Key Words: Head and neck squamous cell carcinoma, tropomyosin-related kinase B, Brain-derived neurotrophic factor, invasiveness.

Table I. *Origin of tissue samples used in the study.*

HNSCC sample #	Origin of specimen	TNM	Grading	Age of patient (years)	Gender
1179	Larynx	T3N1M0	G2	44	m
1164	Larynx	T4N2M0	G2	75	m
1194	Larynx	T3N2M1	Unknown	81	m
1224	Oropharynx	Unknown	G3	57	m
1347	Larynx	T3N3M1	G2	68	f
1372	Oropharynx	T4N2Mx	G2	52	m
1389	Hypopharynx	T4N2M0	G2	52	m
1409	Hypopharynx	T2N2M0	G2	55	m
1228	Hypopharynx	T1N0M0	G2	63	m
1480	Hypopharynx	T2N2Mx	Unknown	48	m
1379	Hypopharynx	T2N0M0	G2	65	m
1303	Oropharynx	T2N2M0	G2	53	f
1433	Hypopharynx	T2N2M0	G3	67	m
1263	Oropharynx	T4N2M0	G2	76	m
1208	Larynx	T3N0M0	Unknown	64	m
1427	Larynx	T3N1M1	Unknown	57	m
1414	Larynx	T2N2M0	G2	67	f
1248	Larynx	T4N2M0	G2	75	m
1261	Hypopharynx	T2N1M0	G3	71	f
1397	Hypopharynx	T4N3M0	G3	44	f
1229	Hypopharynx	T2N2M0	Unknown	53	f
1266	Oropharynx	T3N2M0	Unknown	52	m
1237	Larynx	T2N1M0	G3	52	m

TNM=tumor, node, metastasis (classification according to the guidelines of the International Union Against Cancer). G=grading, G1: well-differentiated, G2: moderate and G3: low differentiation; m=male; f=female.

Materials and Methods

HNSCC tissue samples were obtained during regularly scheduled tumor surgery after informed consent of the patient (Table I). Soft palate control tissue was obtained from regularly scheduled uvulopalatopharyngoplasty (UVPP) surgery after informed consent. HNSCC cell lines used are as described in Table II.

Cell culture. Cells were grown in Dulbecco's modified Eagle's medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal calf serum (Biochrom AG, Berlin, Germany), penicillin and streptomycin at 37˚C and 5% CO_2. The squamous cell carcinoma cell lines UM- and UT- were as previously described by T.E. Carey (University of Michigan, MI, USA) and R. Grénman (University of Turku, Finland) respectively (10). The UMB- (University of Marburg) cell lines (UMB-SCC-745, UMB-SCC-864, UMB-SCC-969) were as described elsewhere (11) (Table II).

Antibodies. Anti-TrkB and secondary FITC- and HRP-coupled anti-rabbit and anti-mouse antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-β-Actin antibodies were from Sigma (St. Louis, MO, USA). Biotinylated goat anti-rabbit antibodies were from Dako (Dako Deutschland GmbH, Hamburg, Germany).

Immunocytochemistry. Cells were grown on cover slides in 6-well tissue culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany) until 50% confluent. They were then fixed with methanol (–20˚C) and stained with the anti-TrkB antibody (1:250) in a buffer consisting of 3% bovine serum albumin and 0.3% Nonidet P40 in phosphate-buffered saline (pH 7.4) for one hour. Subsequently the cells were washed and incubated with the secondary FITC-coupled anti-rabbit antibody (1:250) for an additional 45 minutes. Negative controls (secondary antibody alone) did not show any significant background staining. The resulting FITC-signal was visualized by confocal laser scanning microscopy (Fluoview, Olympus Deutschland GmbH, Hamburg, Germany) to assess the cellular expression of the TrkB receptor.

Immunohistochemistry. Immunohistochemical detection of TrkB was performed using the ABC (avidin-biotin-complex) method. Representative slices of 3-μm thickness were generated from HNSCC tumor samples using a microtome from Leica (Leica Microsystems AG, Wetzlar, Germany). Slides were evaluated with the Ax70 microscope (Olympus Deutschland GmbH, Hamburg, Germany) for the expression of TrkB.

SDS-PAGE and Western blot analysis. Cells and tissue were washed twice in cold PBS and resuspended in lysis buffer (1% Nonidet P40, 137 mM NaCl, 2 mM ethylene diamine tetracetic acid, 20 mM Tris/HCl (pH7.5), 10% glycerol), supplemented with protease and phosphatase inhibitors (Sigma-Aldrich Corp., Saint Louis, MO, USA). SDS-PAGE and Western blot analysis were performed under standard conditions, using 35 μg of whole cell lysate protein per lane (11). In short, nitrocellulose membranes were blocked with 3% milk/PBS and incubated with the anti-TrkB antibody (1:500) overnight at 4˚C. Membranes were washed thrice for 10 min in 3%

Table II. *Origin of HNSCC cell lines used in the study.*

Name	Origin of primary	Origin of specimen	TNM	Grading	Age of patients (years)	Gender
UM-SCC-1	Floor of mouth	Primary tumor site	T2N0M0	G2	73	m
UM-SCC-2	Alveolar ridge	Primary tumor site	T2N0M0	G1	63	f
UM-SCC-3	Nasal columella	Lymph node metastasis	T1N0M0	G1-G2	73	f
UM-SCC-4	Tonsillar	Primary tumor site	T3N2aM0	G3	47	f
UM-SCC-9	Anterior tongue	Primary tumor site	T2N0M0	G1-G2	71	f
UM-SCC-14C	Floor of mouth	Primary tumor site	T1N0M0	G3	58	f
UM-SCC-19	Bottom of the tongue	Primary tumor site	T2N1M0	G2-G3	67	m
UM-SCC-22B	Hypopharynx	Lymph node metastasis	T2N1M0	G2	58	f
UM-SCC-27	Anterior tongue	Lymph node metastasis	T1N0M0	Unknown	62	m
UMB-SCC-745	Oropharynx	Primary tumor site	T4N2M0	G2	48	m
UMB-SCC-864	Tongue	Primary tumor site	T2N2M0	G2	59	m
UMB-SCC-969	Tonsil	Primary tumor site	T4N2M1	G2	67	m
UT-SCC-8	Supraglottic larynx	Primary tumor site	T2N0M0	G1	42	m
UT-SCC-10	Tongue	Primary tumor site	T1N0M0	G2	62	m
UT-SCC-12A	Skin of the nose	Primary tumor site	T2N0M0	G1	81	f
UT-SCC-16A	Tongue	Primary tumor site	T3N0M0	G3	77	f
UT-SCC-16B	Tongue	Lymph node metastasis	T3N0M0	G3	77	f
UT-SCC-19A	Glottic larynx	Primary tumor site	T4N0M0	G2	44	m
UT-SCC-19B	Glottic larynx	Primary tumor site	T4N0M0	G2	44	m
UT-SCC-24A	Anterior tongue	Primary tumor site	T2N0M0	G2	41	m
UT-SCC-26A	Hypopharynx	Lymph node metastasis	T1N2M0	G2	60	m

TNM=tumor, node, metastasis (classification according to the guidelines of the International Union Against Cancer). G=grading, G1: well differentiated, G2: moderate and G3: low differentiation; m=male; f=female.

milk/PBS and then were incubated with an HRP-coupled anti-rabbit antibody (1:1000) for 1 hour. Membranes were washed and bands were visualized on x-ray film (Agfa, Cologne, Germany) using the enhanced chemiluminescence method (Amersham, Buckinghamshire, United Kingdom).

Measurement of cellular invasiveness. MDCK-C7 and A7 melanoma cells are as described by Zak *et al.* (12). Evaluation of cellular invasiveness was performed as described elsewhere (13). In short, MDCK-C7 cells were grown on filter membranes with a pore size of 0.4 μm and a culture surface of 4.25 cm^2 (ThinCerts™, Greiner Bio-One, Frickenhausen, Germany). Transepithelial electrical resistance (TEER) was measured over the subsequent days using an epithelial voltohmmeter (EVOM™, World Precision Instruments, Sarasota, FL, USA) until reaching values around 8 $k\Omega cm^2$ (Figure 2). A defined number of test cells ($5x10^5$) was applied on top of a MDCK-C7 monolayer. The same MDCK-C7 cells were used as a negative control, whereas A7-melanoma cells served as a positive control. In all experiments, the same cell numbers were used for the tested tumor cell lines and the controls. At least three independent experiments were carried out for each cell line. TEER breakdown will occur if the integrity of the MDCK monolayer is compromised due to loss of tight junction adhesion. The HNSCC cell lines UM-SCC-1, -2, -19, UMB-SCC-969 and UT-SCC-10 (Table II) expressed the highest levels of TrkB and therefore were selected for measurement of cellular invasiveness. Since the primary interest of this experiment was to look for an overall effect of BDNF on cellular invasiveness, the results for the 5 tested HNSCC cell lines were pooled together and are further referred to as "HNSCC cell lines" (Figure 2).

Results

TrkB is overexpressed in HNSCC tissues. To evaluate the degree of TrkB protein expression in HNSCC tissues (Table I), Western blot analysis was performed on total tumor tissue lysates. As seen in Figure 1A (top) most tissues express detectable levels of TrkB protein that is present in two isoforms, a full length (TrkB.FL) and a smaller truncated (TrkB.T) form. Two tissues expressed surprisingly high levels of TrkB.T (Figure 1A, top; samples 1379 and 1303). In sharp contrast, there was no significant signal in the control tissue, representing normal mucosa of the soft palate (Figure 1A, top). As expected, the positive control (rat brain) exhibited a distinct TrkB signal. Actin was used as an internal control in all Western blot analyses to verify similar protein loads (not shown). Immunohistochemical staining of HNSCC tissues shows distinct TrkB expression in tumor cells (Figure 1A, bottom). Tissue #1379 was chosen as an example for TrKB expression in HNSCC tissues since expression of the receptor was particularly high in this tissue. However, TrkB was also detectable by immunohistochemistry in tissues that expressed lower TrkB protein levels (not shown).

TrkB is overexpressed in HNSCC cell lines. TrkB expression was further determined in HNSCC cell lines (Table II).

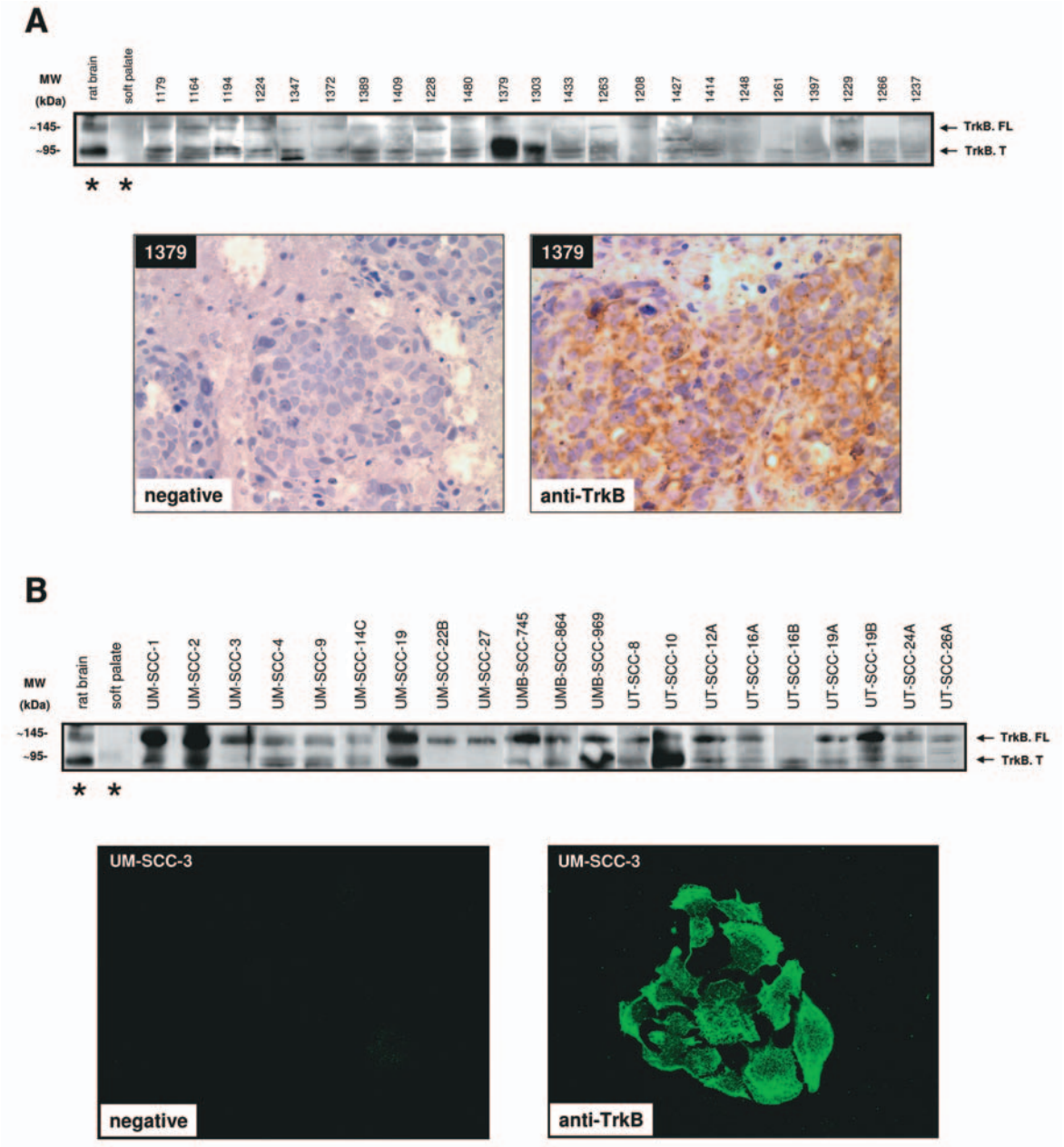
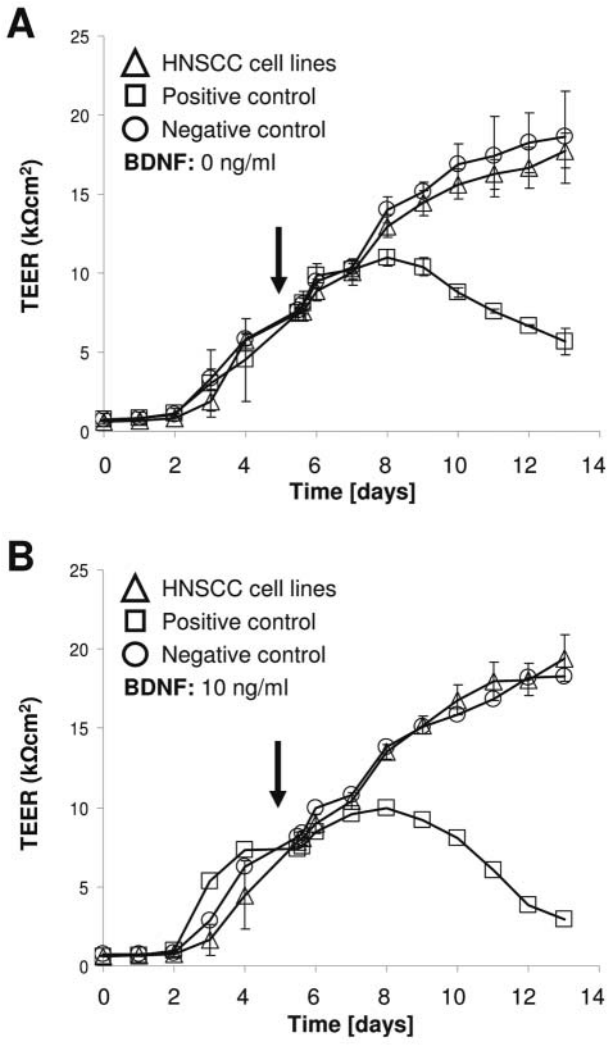


Figure 1. *The receptor tyrosine kinase TrkB is overexpressed in HNSCC tissues and cell lines. TrkB expression of HNSCC tissues (Table I) was evaluated by Western blot analysis (A, top) and immunohistochemistry (A, bottom) as described in Materials and Methods. Similarly, TrkB expression was also determined in HNSCC cell lines (Table II) by Western blot analysis (B, top) and immunocytochemistry (B, bottom). *The same controls are shown for HNSCC tissues and cell lines (rat brain, positive control; soft palate, normal tissue control; TrkB.FL, full length TrkB; TrkB.T, truncated TrkB).*

Most of the tested cell lines exhibited a strong TrkB signal (Figure 1B, top panel). Frequently, overexpression of TrkB also included the full length TrkB.FL isoform. Positive and negative controls were the same as described for HNSCC tissues. A representative immunocytochemical staining of UM-SCC-3 cells shows an intense cellular TrkB-signal (Figure 1B, bottom panel).

Stimulation of HNSCC cells with the TrkB-ligand BDNF does not affect cellular invasiveness. HNSCC cell lines were treated with 10 or 100 ng/ml BDNF as described in the Materials and Methods section. There was no change in electrical resistance compared to the negative control (MDCK cells alone) after addition of HNSCC tumor cells and treatment with BDNF, whereas after addition of A7-



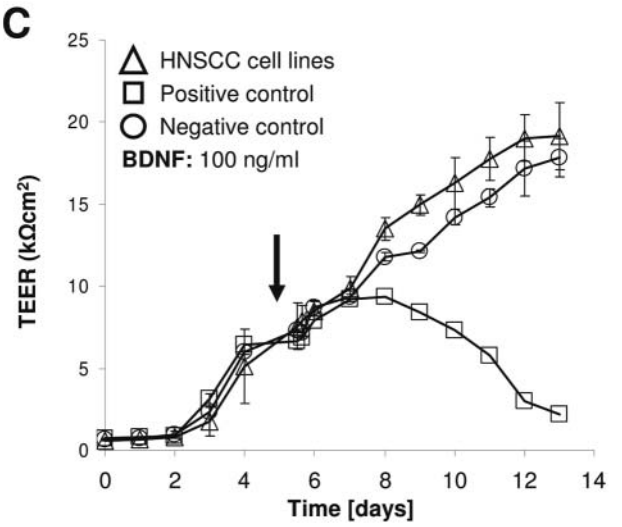


Figure 2. *Stimulation of TrkB-expressing HNSCC cell lines with the TrkB-specific ligand BDNF does not affect HNSCC cell invasiveness. To evaluate changes in the invasiveness of TrkB-expressing HNSCC cell lines after BDNF-stimulation, five HNSCC cell lines (UM-SCC-1, -2, -19, UMB-SCC-969 and UT-SCC-10) were stimulated with 10 or 100 ng/ml BDNF as described in Materials and Methods. A7-melanoma cells were used as a positive and MDCK-C7 cells as a negative control. Cells were added on top of the MDCK-C7 cell barrier when the TEER reached ~8 kΩcm² (arrow) (A-C). The TEER was measured during the subsequent days.*

melanoma cells (positive control) a pronounced breakdown in transepithelial electrical resistance (TEER) occurred 2 to 3 days after the addition of the cells (Figure 2A-C).

Discussion

Normal epithelial cells require attachment at their basal side to a basement membrane or a similar support to prevent apoptosis. The specific form of apoptosis that occurs after a cell loses attachment to its support is called anoikis. Epithelial tumor cells usually are resistant to anoikis, enabling them to survive outside of the cell layer (2). In a previous study, Douma and co-workers were looking for genes associated with anoikis-resistance (3). They used normal rat intestinal epithelial cells and infected them with a retroviral cDNA library of candidate genes. They further observed which clones could grow despite lack of attachment support, *i.e.* which clones were resistant to anoikis. Then they recovered and identified the associated gene of the anoikis-resistant clones. As a master regulator of anoikis-resistance of rat intestinal epithelial cells, the receptor tyrosine kinase tropomyosin-related kinase B (TrkB) was identified. Interestingly, TrkB is a receptor tyrosine kinase, previously isolated from mouse neural tissues and being implicated in neural development (4, 5). Douma and co-workers very impressively demonstrated that TrkB acts as an essential factor for metastatic spread of epithelial cells since only the TrkB-expressing epithelial cells resulted in massive metastatic spread. In sharp contrast, there was no metastatic spread observed after using the respective TrkB-negative control cells. Moreover, it was shown that the observed effect could be enhanced after cotransfection of brain-derived neurotrophic factor (BDNF), the natural ligand of TrkB (3, 8). Interestingly, there is growing evidence that TrkB plays a role in the survival not only of solid tumors such as neuroblastoma, prostate cancer, basal cell carcinoma and squamous cell carcinoma of the skin (7, 8) but also of non-solid tumors such as multiple myelomas (9).

The study by Douma and co-workers prompted us to look for TrkB expression in head and neck squamous cell carcinoma (HNSCC). This epithelial tumor represents the major malignancy of the upper aero-digestive tract (1). In

our study, we demonstrated that TrkB is significantly overexpressed in HNSCC tissues and cell lines compared to normal mucosa. Douma *et al.* already demonstrated that TrkB expression renders tumor cells resistant to anoikis (3). To further investigate, if tumor cells also become more invasive, we tested the invasiveness of TrkB-expressing HNSCC cell lines before and after addition of BDNF (Figure 2). Using the electrical resistance breakdown assay, we previously demonstrated that HNSCC cell lines exhibit rather low invasiveness levels *in vitro* compared to other cell lines such as melanoma cells (13). The tested HNSCC cell lines exhibited a very low invasiveness even after stimulation with BDNF, the natural ligand of TrkB. We therefore concluded that the observed ability of TrkB-dependent metastatic spread is likely due to suppression of anoikis and survival of tumor cells rather than due to enhanced invasiveness of the tumor cells. In what way TrkB could represent a promising therapeutic target is unknown since the exact biological function of this receptor, is still unclear. In a study by Ho *et al.* (14), it was observed that TrkB-expressing neuroblastoma cells became more sensitive to chemotherapy after inhibition with the Trk-specific inhibitor CEP-2563. In a phase I clinical trial conducted on 18 patients, Undevia *et al.* (15) observed a low toxicity of the Trk inhibitor CEP-2563. However, up to this point there is no clear information about the anti-tumour efficacy of such Trk inhibitors.

To summarize, in our study we showed that TrkB is significantly overexpressed in HNSCC tissues and cell lines and that expression or ligand stimulation of the receptor does not render cell lines more invasive. TrkB therefore appears to play a significant role in HNSCC disease presumably by rendering HNSCC cells resistant to anoikis as demonstrated earlier by Douma and coworkers (3). Future studies should investigate potential therapeutic benefits of TrkB inhibition in the treatment of HNSCC disease.

Acknowledgements

We thank Ms. Roswitha Peldszus and Ms. Maria Sadowski for technical assistance, and Dr. Bette and Prof. Weihe (Department of Anatomy and Cell Biology, Philipps University, Marburg, Germany) for using the confocal microscope.

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Received May 16, 2007
Revised June 27, 2007
Accepted July 24, 2007