

ErbB/HER receptor activation and preclinical efficacy of lapatinib in vestibular schwannoma[†]

Sylwia Ammoun*, Clare H. Cunliffe*, Jeffrey C. Allen, Luis Chiriboga, Filippo G. Giancotti, David Zagzag, C. Oliver Hanemann, and Matthias A. Karajannis

Clinical Neurobiology, Peninsula College for Medicine and Dentistry, University of Plymouth, Plymouth, UK (S.A., C.O.H.); Division of Neuropathology (C.H.C., L.C., D.Z.), Department of Neurology (J.C.A.), Division of Pediatric Hematology/Oncology (J.C.A., M.A.K.), New York University School of Medicine, New York, New York; Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York (F.G.G.)

Vestibular schwannomas (VS) arising sporadically or in patients with neurofibromatosis type 2 (NF2) consistently lack expression of Merlin, a tumor suppressor. Conventional treatment options include surgery and radiotherapy but there is no validated medical option. Recent evidence suggests that Merlin deficiency may result in abnormal activation of receptor tyrosine kinases (RTKs) and downstream signaling, promoting tumor growth. Although small-molecule RTK inhibitors are widely available for clinical use, no such therapy has been validated in patients with VS. To screen for RTK activation, surgical VS specimens from patients with and without NF2 were analyzed by phospho-RTK profiling arrays. Downstream signaling pathway activation was analyzed by phospho-MAPK arrays. Activated RTKs and downstream kinases were validated immunohistochemically in corresponding formalin-fixed, paraffin-embedded tissues. Phospho-RTK arrays and immunohistochemistry showed consistent overexpression and activation of EGFR family receptors and

evidence of ERK1/2 downstream signaling was observed in all samples analyzed ($n = 11$). Based on the findings, the small-molecule EGFR/ErbB2 kinase inhibitor lapatinib was selected for evaluation of target inhibition and treatment efficacy in our *in vitro* human schwannoma model. EGFR/ErbB2 targeted therapy with lapatinib inhibited ErbB2 phosphorylation and survivin upregulation, as well as downstream ERK1/2 and AKT activation, resulting in decreased proliferation. We conclude that EGFR family receptor activation is a consistent feature of both sporadic and NF2-related VS. Molecular targeted therapy with lapatinib downregulates survivin and has antiproliferative activity in a preclinical VS model. Based on these findings, a clinical trial with lapatinib for the treatment of VS is currently underway.

Keywords: EGFR, ErbB2, receptor tyrosine kinases, targeted therapy, vestibular schwannoma.

Received September 21, 2009; accepted December 7, 2009.

[†]This paper was presented in part at the International Society of Pediatric Neuro-Oncology 2008 Meeting, Chicago, IL, and the Children's Tumor Foundation 2009 NF Conference, Portland, OR.

*These authors contributed equally to this work.

Corresponding Authors: Matthias A. Karajannis, MD, MS, Division of Pediatric Hematology/Oncology and NYU Cancer Institute, Hassenfeld Children's Center for Cancer and Blood Disorders, 160 East 32nd Street, 2nd Floor, New York, NY 10016 (matthias.karajannis@nyumc.org); C. Oliver Hanemann MD, PhD, FRCP, Clinical Neurobiology, Peninsula College for Medicine and Dentistry, John Bull Building, Research Way, Tamar Science Park, Plymouth PL6 8BU (oliver.hanemann@pms.ac.uk).

Vestibular schwannomas (VS) usually arise from Schwann cells surrounding the vestibular branch of the cochlear nerve. Sporadic unilateral VS is the most frequent presentation, usually with hearing impairment, tinnitus, and vertigo in the 5th or 6th decade of life, but patients with neurofibromatosis type 2 (NF2) typically present with bilateral VS at an earlier age. The treatment of sporadic VS is mainly surgical or radiosurgical, and increased hearing loss and facial nerve impairment are significant risks of treatment. The morbidity of these therapies in patients with NF2 is frequently greater, as related to the larger size of tumors at presentation. In addition, VS frequently recur in NF2 patients following an incomplete resection. For NF2

patients, it would be highly desirable to develop less invasive medical options that are effective not only for VS, but also for other schwannomas and the frequently associated meningiomas and spinal cord ependymomas.¹

Both sporadic and NF2-related VS are deficient in Merlin, a tumor suppressor. Merlin is closely related to the ezrin/radixin/moesin family of cytoskeletal linker proteins, which link membrane-associated proteins to the actin cytoskeleton.^{2,3} Although the mechanisms through which Merlin controls cell proliferation remain poorly understood, recent studies suggest that abnormal activation of receptor tyrosine kinases (RTKs) may play a key role in cell proliferation. Activated Merlin localizes to and stabilizes cadherin-dependent cell-to-cell junctions, which may result in a negative regulatory effect on RTKs at the cell membrane.^{4–6} Studies in *Drosophila* show that Merlin also facilitates endocytic trafficking of membrane receptors, including RTKs.⁷ In addition, Merlin may also directly oppose the activation of Rac, which is recruited through joint integrin-RTK signaling.^{8,9} These mechanisms may, at least in part, explain the observations that loss of Merlin activates pro-survival and proliferation pathways such as ERK1/2, AKT, and JNK.^{8,10–12} Merlin-deficient cells overexpress PDGFR β with downstream activation of ERK1/2 and AKT, resulting in abnormal growth signals.^{10,13} Aberrant EGFR activation due to anomalous receptor compartmentalization has been linked to Merlin deficiency as well, and pharmacological inhibition of EGFR signaling successfully blocked cellular proliferation in Merlin-negative cells.¹⁴

These findings suggest that merlin affects multiple signaling pathways. The data, however, originates from multiple cell types and *in vitro* systems, and the pathways most relevant for VS growth *in vivo* are not known.

In line with *in vitro* observations, expression of several ErbB family receptors, including EGFR, has been reported in surgical schwannoma samples.¹⁵ In another study, an anti-ErbB2 monoclonal antibody reduced schwannoma cell proliferation *in vitro*.¹⁶ More recently, an EGFR inhibitor and an ErbB2 antibody were shown to reduce the growth of VS in a xenograft model.¹⁷ Taken together, these findings suggest that abnormal signaling via EGFR, ErbB2, and PDGFR β may represent attractive therapeutic targets in VS. Although a plethora of novel agents targeting RTK signaling pathways are under clinical investigation,¹⁸ no such drugs have been validated for the treatment of VS. A recent case report suggests that EGFR-directed therapy with erlotinib may have activity in patients with NF2-related VS.¹⁹

To date, no comprehensive profiling of RTK expression and activation has been performed in VS. To screen for potential therapeutic targets, surgical VS specimens from patients with and without NF2 were analyzed by phospho-RTK profiling arrays. Activation of downstream signaling pathways was analyzed by phospho-mitogen-activated protein kinases (MAPK) profiling arrays. Subsequently, activated RTKs and downstream kinases were validated immunohistochemically in corresponding formalin-fixed, paraffin-embedded tissues (FFPE).

Based on our finding of consistent activation of ErbB family receptors and downstream ERK1/2 signaling, we decided to evaluate the efficacy and mechanisms of action of lapatinib, a small molecule that inhibits both EGFR and ErbB2, in our *in vitro* Schwannoma model. Lapatinib effectively inhibited β -heregulin-mediated phosphorylation/activation of ErbB2 receptor, ERK1/2, AKT, and S6 ribosomal protein, as well as cell proliferation. Treatment with lapatinib over 72 hours resulted in decreased expression of the antiapoptotic protein survivin, which is known to be regulated by ErbB2 signaling.^{20,21}

Materials and Methods

Chemical Compounds and Antibodies

Lapatinib was provided by GlaxoSmithKline, dissolved in DMSO at stock concentration of 10 mM, and stored at -20°C . All antibodies and chemicals were purchased from Cell Signaling and Sigma, respectively, unless stated otherwise.

Patient Samples

Following informed consent, fresh surgical tissue specimens were collected from VS patients, and a portion of the tumor not needed for diagnosis was snap frozen immediately and stored in a tumor bank at -70°C . Tumor tissue from 11 VS patients was available for this study (7 NF2 and 4 nonNF2 patients). Frozen specimens were available from 10 patients and FFPE tissue available from 8 patients. Two fresh-frozen specimens of normal facial nerves, including 1 from an NF2 patient, served as controls. All samples were collected and used under protocols approved by the Institutional Review Board at New York University School of Medicine.

Antibody Arrays

To screen RTK phosphorylation status of VS tumors *in vivo*, we analyzed surgical VS specimens from 7 NF2 and 3 nonNF2 patients using a phospho-RTK profiling array, detecting relative phosphorylation levels of 42 RTKs (AXL, DTK, EGFR, ErbB2, ErbB3, ErbB4, EphA1, EphA2, EphA3, EphA4, EphA6, EphA7, EphB1, EphB2, EphB4, EphB6, FGFR1, FGFR2 α , FGFR3, FGFR4, FLT3, HGFR, Insulin R, IGFR1, MER, MSPR, PDGFR α , PDGFR β , SCFR, M-CSFR, C-RET, ROR1, ROR2, Tie-1, Tie-2, TrkA, TrkB, TrkC, MuSK, VEGFR1, VEGFR2, and VEGFR3). To determine the activation status of downstream signaling pathways, we subsequently analyzed the specimens for relative phosphorylation of 18 MAPK and serine/threonine kinases, using a phospho-kinase profiling array (AKT1, AKT2, AKT3, AKT pan, ERK1, ERK2, GSK3 α/β , GSK3 β , HSP27, JNK1, JNK2, JNK3, JNK pan, MSK2, p38 α , p38 β , p38 γ , p38 δ , p70S6K, RSK1, and RSK2). Histopathologically verified samples from

sporadic and NF2-related VS tumors and normal vestibular nerve (controls) were thawed in lysis buffer, homogenized by hand 50 times with disposable pestles, incubated for 30 minutes at 4°C with inversion and then cleared by centrifugation at 13 000 rpm, for 10 minutes at 4°C. Protein concentration was quantified using a BCA Protein assay (Pierce) as per manufacturer's instructions. Phospho-RTK and phospho-MAPK array analysis (ARY-001 and ARY-002 Proteome Profiler, R&D Systems) was performed as per manufacturer's instructions using 500 µg protein lysate per array.

Immunohistochemistry

To validate the phospho-array findings and localize the observed signals histologically, we performed immunohistochemistry on formalin-fixed paraffin-embedded samples from all VS samples with paraffin-embedded tissue available ($n = 8$). We also stained for survivin, an inhibitor of apoptosis (IAP) family protein, which is expressed in a variety of tumors and regulated via activation of EGFR/ErbB2 and PI3K/AKT.^{20–22} Briefly, 5-µm sections were deparaffinized, followed by microwave antigen retrieval. Primary monoclonal antibodies used were: EGFR, ErbB2, phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴, clone 20G11), and survivin (clone 12C4, DAKO).

The DAKO EnVision kit was used for detection and staining as per manufacturer's instructions. Staining was scored as described previously,²³ with (i) weak focal staining, (ii) weak diffuse staining, (iii) strong focal staining, and (iv) strong diffuse staining.

Human Schwann and Schwannoma Cell Isolation and Culture

Human primary Schwann and schwannoma cells were isolated and cultured as previously described.²⁴ For immunoblotting experiments, cells from 5 NF2-related and 1 sporadic schwannomas were used. For proliferation assays, cells from 7 NF2-related and 5 sporadic schwannomas were used.

Immunoblotting

Cells were grown in pre-coated 35 mm plates (Greiner Bio-One), serum starved for 24 hours, stimulated with 100 ng/ml of β -heregulin for 5 minutes, and lysed.²⁵ Lapatinib (10 µM) was added 40 minutes prior to stimulation. Western blotting was performed as previously described,^{6,8} using anti-active-MAPK (anti-pThr¹⁸³-pTyr¹⁸⁵-ERK1/2) (1:2000; Promega), anti-phospho-AKT (Ser⁴⁷³) (1:500), anti-phospho-ErbB2 (Tyr^{1221/1222}) (1:500), anti-phospho-S6 ribosomal protein (Ser^{235/236}) (1:500), and anti-survivin (1:500) primary antibodies. The detection of the primary antibodies was performed using goat-anti-rabbit HRP-conjugated secondary antibodies (Biorad). Western blot bands were detected by ECL-plus (Amersham). Rho-GDI was used as a loading control and detected

by anti-RhoGDI antibody (1:500, Santa Cruz Biotechnology).²⁶ FluorS-Multi-Imager (Bio-Rad) was used to measure band densities.

Nuclear Staining for Cell Growth

Cells were cultivated on pre-coated 96-well plates (Nunc) for 24 or 72 hours and fixed with 4% paraformaldehyde. Nuclei were stained with DAPI (1 µg/ml) for 10 minutes and total cell numbers were manually counted. Cell viability determination was performed using propidium iodide (PI) (2.5 µg/ml). Lapatinib (3, 5, and 10 µM) was added 40 minutes before cell stimulation.

Data Analysis

Student's 2-tailed *t*-tests were applied. Every experiment was repeated 3 times at minimum and at least 3 independent batches of cells from different individuals were tested. In cell proliferation and viability assays, all cells in each well were counted (ns means not significant, $P > 0.05$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$). In figures, mean \pm SEM is given.

Results

Phospho-RTK Profiling Arrays

Fresh-frozen specimens from 7 NF2 patients and 3 nonNF2 patients were available for analysis. An example of a phospho-RTK array from a VS sample is shown (Fig. 1A) and results from all samples are summarized (Table 1). Relative phosphorylation signal of each kinase was recorded as strong, weak, or undetectable. All VS specimens showed phosphorylation of several RTKs, with EGFR family receptors being most strongly and consistently activated; all VS specimens showed activation of EGFR and at least 1 additional EGFR family receptor (ie, ErbB2, ErbB3, and/or ErbB4). Additional RTKs detected and phosphorylated in <50% of the samples were PDGFR β , AXL, SKY, MER, and RON. No signals were detectable in the control specimens (Fig. 1B and Table 1).

Phospho-MAPK Profiling Arrays

Analysis of a VS specimen by phospho-MAPK array is shown (Fig. 2A). Relative phosphorylation signal of each kinase was recorded as strong, weak, or undetectable (Table 2). Strong phosphorylation of ERK1/2 was detected in all samples analyzed. Weak phosphorylation of several p38 kinase subunits was present in most samples, as well as GSK3 α/β , HSP27, JNK2, and RSK1 (p90 ribosomal S6 kinase) in some. Of note, no phosphorylation of AKT or p70 S6 kinase (p70 S6K, a biological readout of mTOR pathway activation) was visible. In the control specimens, no signals were detectable (Fig. 2B and Table 2).

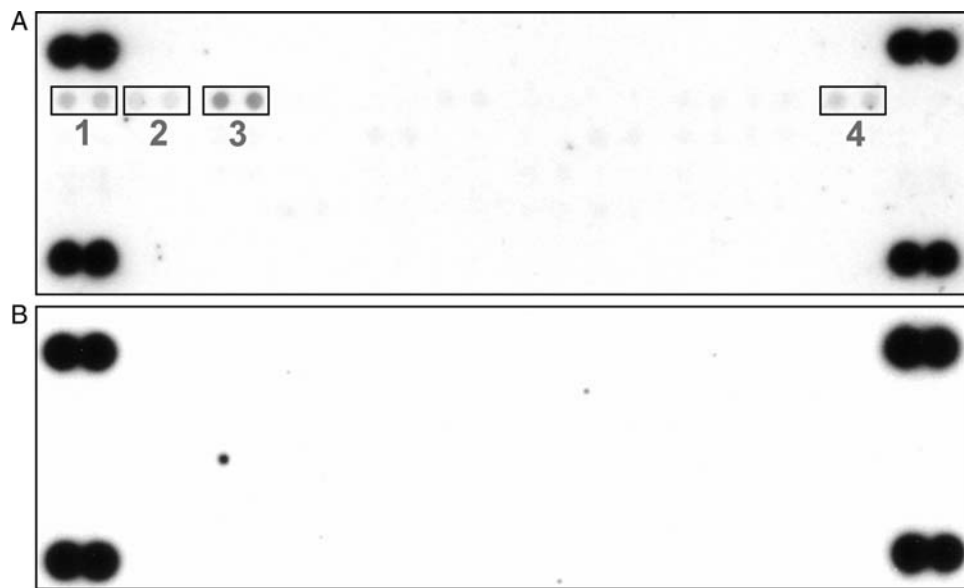


Fig. 1. Phospho-RTK profiling arrays. Representative arrays from a VS sample (A) and a control sample (normal vestibular nerve, B), showing relative phosphorylation status of RTKs. Phosphorylation level of each RTK is represented by duplicate spots. Positive control spots are located in all 4 corners of the array. (1) EGFR, (2) ErbB2, (3) ErbB3, (4) AXL. Results from all samples are summarized in Table 1.

Table 1. Phospho-RTK profiling array results

Patient	NF2 status	EGFR	ErbB2	ErbB3	ErbB4	PDGFR β	AXL	SKY	MER	RON
1	NF2	++	—	—	+	—	—	—	+	+
2	NF2	++	+	+	—	+	—	—	—	+
3	NF2	++	+	+	—	—	—	—	—	—
4	NF2	+	+	++	—	+	—	—	—	+
5	NF2	+	+	++	—	+	—	++	—	—
6	NF2	++	—	—	+	—	+	+	—	+
7	NF2	+	+	++	—	—	+	—	—	—
8	Sporadic	+	—	—	+	—	—	++	—	—
9	Sporadic	+	+	+	—	—	—	+	—	—
10	Sporadic	+	+	++	—	+	+	—	—	—
11	Sporadic	NA	NA	NA	NA	NA	NA	NA	NA	NA
Control	NF2	—	—	—	—	—	—	—	—	—
Control	Sporadic	—	—	—	—	—	—	—	—	—

Phosphorylation status for each kinase is indicated as strong (++), weak (+), or undetectable (—). Abbreviation: NA, denotes appropriate tissue not available for testing.

Immunohistochemistry

In keeping with the phospho-array results, immunohistochemistry confirmed expression of EGFR and ErbB2, as well as phosphorylation of ERK1/2 in tumor cells of all samples tested (Fig. 3A, B, and C, respectively). In addition, expression of survivin was detected in the tumor cells of all patients tested (Fig. 3D). Immunohistochemistry results are summarized in Table 3. Adjacent connective and normal nerve tissues did not stain positive for any of the antigens probed. Additional negative controls without primary antibodies showed no staining (not shown). Positive controls included lung and breast cancer specimens with known antigen expression.

Taken together, the results indicated that overexpression and phosphorylation of EGFR and ErbB2, as well as

downstream activation of ERK1/2 are consistently observed in both sporadic and NF2-related VS in vivo. In addition, survivin, an anti-apoptotic protein that may be regulated via EGFR/ErbB2 signaling, is overexpressed by VS. Therefore, we tested the “drugability” of the identified molecular targets with lapatinib, a combined EGFR/ErbB2 inhibitor, using a human schwannoma in vitro model.

Immunoblotting

Stimulation of serum-starved human primary schwannoma cells (NF2^{+/+}) for 5 minutes with 100 ng/ml β -heregulin resulted in strong activation/phosphorylation of the ErbB2 receptor followed by increased

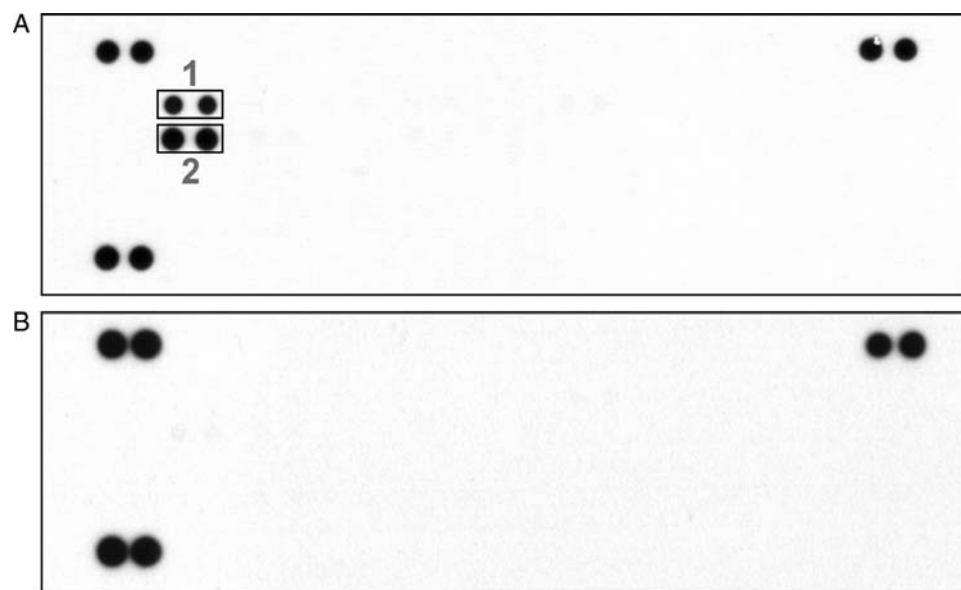


Fig. 2. Phospho-MAPK profiling arrays. Representative arrays from a VS sample (A) and a control sample (normal vestibular nerve, B), showing relative phosphorylation status of MAP kinases and serine/threonine kinases. Phosphorylation level of each RTK is represented by duplicate spots. Positive control spots are located in 3 corners of the array. (1) ERK1, (2) ERK2. Results from all samples are summarized in Table 2.

Table 2. Phospho-MAPK profiling array results

Patient	NF2 status	ERK1	ERK2	p38 α	p38 γ	p38 δ	GSK3 α/β	HSP27	JNK2	RSK1
1	NF2	++	++	–	–	–	+	+	–	–
2	NF2	++	++	+	+	+	+	+	–	–
3	NF2	++	++	+	–	–	–	–	–	–
4	NF2	++	++	+	–	–	+	–	–	+
5	NF2	++	++	–	–	–	–	–	+	+
6	NF2	++	++	+	–	–	–	+	–	–
7	NF2	++	++	–	–	–	–	–	–	–
8	Sporadic	++	++	–	–	–	–	–	–	–
9	Sporadic	++	++	+	–	–	–	+	–	–
10	Sporadic	++	++	+	–	–	–	+	–	–
11	Sporadic	NA	NA	NA	NA	NA	NA	NA	NA	NA
Control	NF2	–	–	–	–	–	–	–	–	–
Control	Sporadic	–	–	–	–	–	–	–	–	–

Phosphorylation status for each kinase is indicated as strong (++), weak (+), or undetectable (–). Abbreviation: NA, denotes appropriate tissue not available for testing.

phosphorylation of ERK1/2, AKT, and S6 ribosomal protein (Fig. 4A–D). Lapatinib at the concentration of 10 μ M completely inhibited β -heregulin mediated but not basal (nonstimulated) ErbB2 receptor, ERK1/2, AKT, and S6 ribosomal protein phosphorylation (Fig. 4A–D). Additionally, long-term (72 hours) treatment of schwannoma cells with lapatinib (5 μ M) led to downregulation of survivin, a human IAP protein (Fig. 4E).

(0.5 μ M) for 24 hours resulted in increased total cell number (determined by DAPI staining of the nuclei) by up to ~20%, which was completely reverted by lapatinib (3 μ M) (Fig. 5A). Cell viability was not affected at these conditions as estimated by PI staining (Fig. 5B). Lapatinib at 5 and 10 μ M significantly decreased schwannoma cell viability in a concentration-dependent manner starting at 24 hours (Fig. 5C).

Proliferation and Cell Viability Assays

The stimulation of human primary schwannoma cells with 100 ng/ml β -heregulin together with forskolin

Discussion

Schwannomas are the third most common tumors of the nervous system, and there is a medical need to find

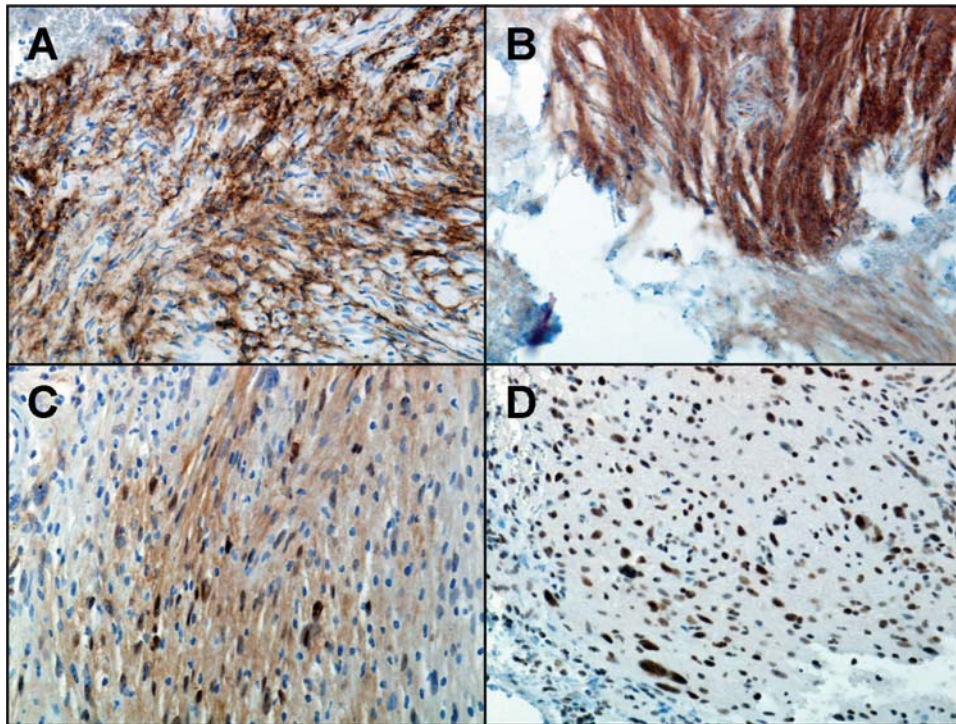


Fig. 3. Immunohistochemistry of VS specimens. Representative staining of VS for EGFR (A), ErbB2 (B), phospho-ERK1/2 (C), and survivin (D) is shown. Antigens are visualized by brown membranous and/or nuclear staining.

Table 3. VS immunohistochemistry results

Patient	NF2 status	EGFR	ErbB2	p-ERK1/2	Survivin
1	NF2	NA	NA	NA	NA
2	NF2	NA	NA	NA	NA
3	NF2	1	1	1	4
4	NF2	1	1	1	4
5	NF2	3	1	NA	3
6	NF2	3	3	1	4
7	NF2	NA	NA	NA	NA
8	Sporadic	3	2	1	3
9	Sporadic	3	2	1	3
10	Sporadic	3	2	3	4
11	Sporadic	3	3	3	4

Staining is scored with (1) weak focal staining, (2) weak diffuse staining, (3) strong focal staining, and (4) strong diffuse staining. Abbreviation: NA, denotes appropriate tissue not available for testing.

specific and effective therapies, especially when they occur in multiple locations such as in NF2. To screen for suitable therapeutic targets, we determined the expression and activation of different RTKs and their downstream signaling pathways that are potentially involved in the development and progression of human schwannoma using phospho-protein profiling arrays. Positive targets were further verified by immunohistochemistry. Based on these data and rational target selection, we employed our primary human *in vitro* schwannoma model to test the molecular target

inhibition and antitumor efficacy of the EGFR/ErbB2 small-molecule inhibitor lapatinib.

We show overexpression and activation of a variety of RTKs previously shown to be overexpressed and activated in VS, including PDGFR and EGFR family RTKs,^{10,13,16,27} as well as additional tyrosine kinase receptors not previously associated with VS, such as AXL, SKY, MER, and RON. The AXL subfamily of RTKs is expressed in a variety of normal tissues and includes AXL (ubiquitously expressed in many organs), SKY (widely expressed in the brain), and MER (monocytes, epithelial cells). However, AXL subfamily RTKs also possess transforming activity and are overexpressed in some cancers.²⁸ Additionally, MER displays antiapoptotic effects and its signaling is directly connected to integrins, src, and FAK.²⁸ Similarly, SKY also signals via src. SKY also interacts with the p85 subunit of PI3K and modulates ERK activity. RON may be found in normal epithelial cells, as well as macrophages, bone marrow stem cells, and osteoclasts,²⁹ but is overexpressed in malignant tumors³⁰ and may promote cell migration, invasion,³¹ and proliferation.³² Since AXL, SKY, MER, and RON are implicated in tumorigenesis and signal via pathways known to be upregulated in human schwannomas (ie, FAK, PI3K, and ERK),^{13,33} the potential role of this group of RTKs in schwannoma development should be investigated in future studies.

In our study, the strongest and most consistent phosphorylation was found for the EGFR family receptors EGFR, ErbB2, ErbB3, and ErbB4, as well as downstream protein kinases such as ERK1/2, p-38 MAPK

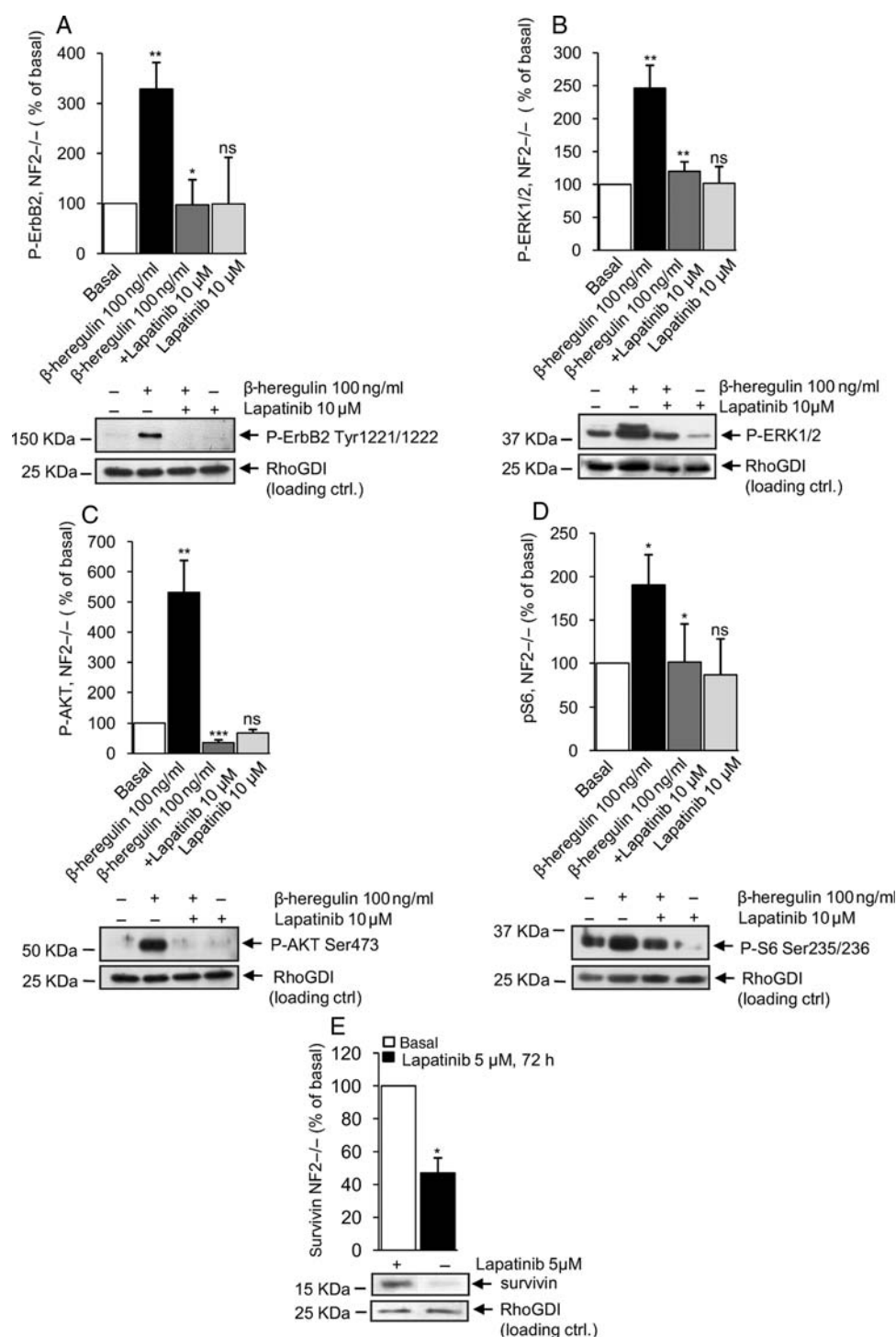


Fig. 4. The effects of lapatinib on β -herregulin-mediated signaling and survivin expression in human primary schwannoma cells (NF2^{-/-}). (A) Lapatinib (10 μ M) is a very potent inhibitor of ErbB2 receptor activity. (B, C, and D) Lapatinib (10 μ M) completely inhibits β -herregulin (100 ng/ml) mediated ERK1/2, AKT, and S6 ribosomal protein phosphorylation/activation. The cells were starved for 24 hours before stimulation, pre-treated with lapatinib (10 μ M) for 40 minutes, and stimulated with β -herregulin (100 ng/ml) for 5 minutes. The phosphorylated/activated levels of ErbB2 p-Tyr^{1221/1222}, ERK1/2 p-Thr¹⁸³/p-Tyr¹⁸⁵, AKT p-Ser⁴⁷³, and S6 ribosomal protein p-Ser^{235/236} were detected by immunoblotting. (E) Lapatinib downregulates the expression of survivin in human primary schwannoma cells. The cells were serum starved for 24 hours and incubated with lapatinib for 72 hours. Day 1: the cells were pre-incubated for 40 minutes with 0.5 μ M lapatinib, Day 2: 0.25 μ M lapatinib was added, Day 3: 5 μ M of lapatinib was added. The cells were lysed on day 4, and total levels of survivin were detected by immunoblotting using anti-survivin antibody. The data are corrected to a loading control RhoGDI and given as a % of basal (nonstimulated cells).

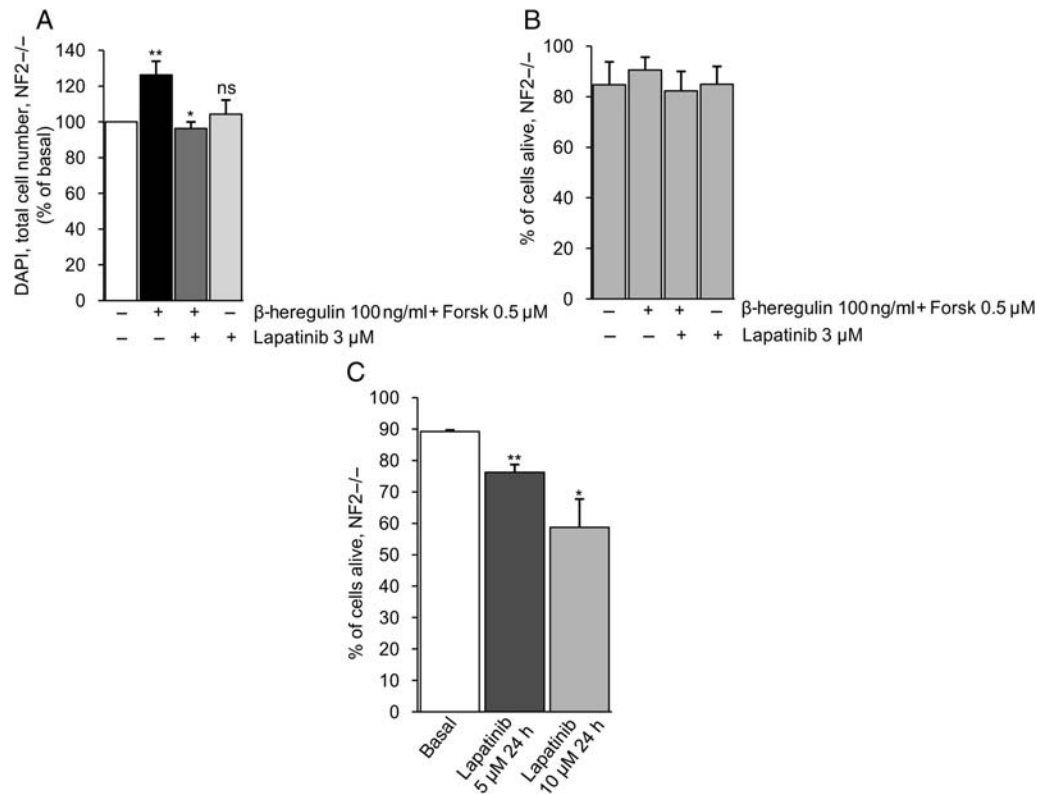


Fig. 5. Lapatinib effects on the proliferation and viability of human primary schwannoma cells (NF2^{-/-}), as well as survivin expression. (A) Lapatinib (3 μM) completely inhibits β-hergulin (100 ng/ml) and forskolin (0.5 μM) mediated schwannoma proliferation. (B) Lapatinib at 3 μM has no significant effect on schwannoma cell viability. (C) Lapatinib at 5 and 10 μM significantly decreases schwannoma cell viability in a concentration-dependent manner after 24 hours treatment. In all experiments, the cells were cultured for 24 hours with or without β-hergulin (100 ng/ml) + forskolin (0.5 μM) and lapatinib. Total cell amount was estimated by nuclear staining with DAPI (1 μg/ml) and cell counting. The viability of the cells was estimated with PI (2.5 μg/ml) staining and cell counting. The data are corrected to a loading control RhoGDI and given as the % of basal (nonstimulated). In (A), the data are normalized to the basal levels (nonstimulated cells) and given as a % of basal (nonstimulated). In (B) and (C), the data are given as a % of total cell number.

(α, β, and δ), and GSK3α/β in both NF2-related and sporadic VS using phospho-protein arrays. The lack of detectable phospho-AKT is consistent with recent biological studies suggesting that mTORC1 is constitutively activated in Merlin-deficient tumors and involved in a negative feedback loop that diminishes PI3K-AKT signaling in response to growth factor stimulation.^{34,35} However, we did not detect activation of p70 S6K by phospho-arrays, which is downstream of mTORC1 and together with phospho-S6 considered a sensitive biological readout of mTORC1 activation. On the other hand, phosphorylation of S6 has been demonstrated in VS specimens recently,³⁴ and lack of detectable phospho-p70 S6K in our array-based assay may be due to technical limitations. In keeping with the predominant phosphorylation of EGFR family receptors identified by the phospho-arrays, strong overexpression of EGFR and ErbB2 was confirmed by immunohistochemistry in our VS specimens. Our current data regarding the expression and phosphorylation of EGFR, ErbB2, and ErbB3 in VS confirm and expand on previous findings.^{13,15,16,36} The observed lack of EGFR expression and response to its ligand EGF in our primary human *in vitro* schwannoma model¹³ is

possibly due to culture conditions. ErbB2/ErbB3 signaling, however, has long been established to be of key importance for Schwann cell differentiation and proliferation,³⁷ and neuregulin 1 (heregulin), an ErbB2/3 receptor agonist, has been shown to be expressed in VS.^{16,38} We therefore chose to concentrate on the functional relevance of ErbB2/ErbB3 receptor signaling and kinase inhibition *in vitro*.

In accordance with data on the crucial role of ErbB2/ErbB3 signaling in Schwann cells and supporting the relevance of ErbB2/ErbB3 overexpression in schwannomas, we found strong phosphorylation/activation of ErbB2 upon stimulation with β-hergulin, leading to the activation of both mitogenic (ERK1/2 and S6 ribosomal protein) and pro-survival (AKT and survivin) pathways in human primary schwannoma cells. As shown in NF2-deficient mouse Schwann cells, Merlin loss may result in accumulation of ErbB2 and ErbB3 receptors on the cell membrane, leading to the activation of downstream signaling pathways such as ERK and AKT and increased cell proliferation.²⁷ Thus, ErbB2 and its downstream signaling pathways may be relevant in human schwannoma development, rendering ErbB2 an important therapeutic target.

Since both EGFR and ErbB2 represent molecular targets in VS, we chose lapatinib for preclinical evaluation. Lapatinib is a small molecule RTK inhibitor that is orally active and reversibly inhibits EGFR as well as ErbB2,³⁹ blocking phosphorylation and activation of ERK1/2 (phospho-ERK1/2) and AKT (phospho-AKT) in EGFR and/or ErbB2-expressing tumor cell lines and animal xenografts.^{40–42} Depending on tumor cell type, lapatinib has cytostatic or cytotoxic antitumor effects.⁴² Correlation between tumor response and pre-treatment levels of (phospho)-ErbB2 and (phospho)-ERK1/2 has been demonstrated in solid tumors.⁴³ Lapatinib has advantages over other small-molecule EGFR inhibitors (ie, erlotinib), as it targets both EGFR and ErbB2. We demonstrate that lapatinib effectively inhibits heregulin-mediated phosphorylation of ErbB2, ERK, AKT, and S6 ribosomal protein. Heregulin-mediated proliferation of human primary schwannoma cells is also completely reversed to the basal level by 3 μ M lapatinib without affecting cell viability. Higher concentrations of lapatinib (5 and 10 μ M), however, significantly decrease schwannoma cell viability in a concentration-dependent manner, consistent with previous findings in nonmalignant cell lines.⁴² Furthermore, prolonged (72 hours) incubation of schwannoma cells with 5 μ M lapatinib strongly decreased the expression of the anti-apoptotic protein survivin, which has been shown to be regulated by EGFR/ErbB2 and PI3K/AKT signaling.^{20–22} In parallel, we found consistent overexpression of survivin in VS *in vivo*, which is in line with prior observations in schwannomas^{44,45} and suggests that survivin should be explored as a therapeutic target in VS. Survivin-directed immunotherapy approaches, as well as survivin antagonists, have been developed for clinical use in cancer⁴⁶ and are being investigated in early-stage clinical trials.

Complementary to our findings, several supporting lines of evidence suggest lapatinib as an attractive candidate for clinical evaluation in the treatment of VS. Based on preclinical studies⁴⁷ as well as clinical responses in patients with brain metastases,⁴⁸ lapatinib may cross the blood–brain barrier and achieve therapeutic drug concentrations in the CNS. Correlation between tumor response and pre-treatment levels of phospho-ErbB2 and phospho-ERK1/2 has been demonstrated in solid tumors.⁴³ Lapatinib is FDA approved for the treatment of metastatic breast cancer and has been shown to be well tolerated with predictable and manageable side effects.⁴⁹ In addition, pediatric safety and dosing data are available from an ongoing pediatric phase I/II trial by the Children's Oncology Group for refractory CNS malignancies (NCT00095940). This is an important consideration since some of the most severely affected VS patients are children with NF2.

In summary, based on our data and additional supporting evidence, we have identified lapatinib as a promising candidate for clinical evaluation in the treatment of VS. Accordingly, we have initiated a multi-center phase 0 clinical trial for VS patients (NCT00863122) to determine the achievable intratumoral concentration of lapatinib and to assess target inhibition as well as biological activity *in vivo*.

Conflict of interest statement: None declared.

Funding

This work was supported by National Institutes of Health grant NCRR M01RR00096 (M.A.K.), the Pediatric Cancer Foundation (M.A.K.), and internal funding PCMD (C.O.H.).

References

- Evans DG, Huson SM, Donnai D, et al. A clinical study of type 2 neurofibromatosis. *Q J Med.* 1992;84:603–618.
- Rouleau GA, Merel P, Lutchman M, et al. Alteration in a new gene encoding a putative membrane-organizing protein causes neurofibromatosis type 2. *Nature.* 1993;363:515–521.
- Trofatter JA, MacCollin MM, Rutter JL, et al. A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. *Cell.* 1993;75:826.
- Qian X, Karpova T, Sheppard AM, McNally J, Lowy DR. E-cadherin-mediated adhesion inhibits ligand-dependent activation of diverse receptor tyrosine kinases. *EMBO J.* 2004;23:1739–1748.
- Takahashi K, Suzuki K. Density-dependent inhibition of growth involves prevention of Egf receptor activation by E-cadherin-mediated cell-cell adhesion. *Exp Cell Res.* 1996;226:214–222.
- Flaic C, Utermark T, Parkinson DB, Poetsch A, Hanemann CO. Impaired intercellular adhesion and immature adherens junctions in Merlin-deficient human primary Schwannoma cells. *Glia.* 2008;56:506–515.
- Maitra S, Kulikauskas RM, Gavilan H, Fehon RG. The tumor suppressors Merlin and expanded function cooperatively to modulate receptor endocytosis and signaling. *Curr Biol.* 2006;16:702–709.
- Kaempchen K, Mielke K, Utermark T, Langmesser S, Hanemann CO. Upregulation of the Rac1/Jnk signaling pathway in primary human Schwannoma cells. *Hum Mol Genet.* 2003;12:1211–1221.
- Morrison H, Sperka T, Manent J, Giovannini M, Ponta H, Herrlich P. Merlin/neurofibromatosis type 2 suppresses growth by inhibiting the activation of Ras and Rac. *Cancer Res.* 2007;67:520–527.
- Fraenzer JT, Pan H, Minimo L, Jr, Smith GM, Knauer D, Hung G. Overexpression of the Nf2 gene inhibits Schwannoma cell proliferation through promoting Pdgfr degradation. *Int J Oncol.* 2003;23:1493–1500.
- Lim JY, Kim H, Kim YH, et al. Merlin suppresses the Sre-dependent transcription by inhibiting the activation of Ras-Erk pathway. *Biochem Biophys Res Commun.* 2003;302:238–245.
- Hanemann CO. Magic but treatable? Tumours due to loss of Merlin. *Brain.* 2008;131:606–615.
- Ammoun S, Flaic C, Ristic N, Schuld J, Hanemann CO. Dissecting and targeting the growth factor-dependent and growth factor-independent extracellular signal-regulated kinase pathway in human Schwannoma. *Cancer Res.* 2008;68:5236–5245.

14. Curto M, Cole BK, Lallemand D, Liu CH, McClatchey AI. Contact-dependent inhibition of Egr signaling by Nf2/Merlin. *J Cell Biol*. 2007;177:893–903.
15. Stonecypher MS, Chaudhury AR, Byer SJ, Carroll SL. Neuregulin growth factors and their ErbB receptors form a potential signaling network for schwannoma tumorigenesis. *J Neuropathol Exp Neurol*. 2006;65:162–175.
16. Hansen MR, Roehm PC, Chatterjee P, Green SH. Constitutive neuregulin-1/ErbB signaling contributes to human vestibular schwannoma proliferation. *Glia*. 2006;53:593–600.
17. Clark JJ, Provenzano M, Diggelmann HR, Xu N, Hansen SS, Hansen MR. The ErbB inhibitors trastuzumab and erlotinib inhibit growth of vestibular schwannoma xenografts in nude mice: a preliminary study. *Otol Neurotol*. 2008;29:846–853.
18. Krause DS, Van Etten RA. Tyrosine kinases as targets for cancer therapy. *N Engl J Med*. 2005;353:172–187.
19. Plotkin SR, Singh MA, O'Donnell CC, Harris GJ, McClatchey AI, Halpin C. Audiologic and radiographic response of Nf2-related vestibular schwannoma to erlotinib therapy. *Nat Clin Pract Oncol*. 2008;5:487–491.
20. Asanuma H, Torigoe T, Kamiguchi K, et al. Survivin expression is regulated by coexpression of human epidermal growth factor receptor 2 and epidermal growth factor receptor via phosphatidylinositol 3-kinase/Akt signaling pathway in breast cancer cells. *Cancer Res*. 2005;65:11018–11025.
21. Xia W, Bisi J, Strum J, et al. Regulation of survivin by ErbB2 signaling: therapeutic implications for ErbB2-overexpressing breast cancers. *Cancer Res*. 2006;66:1640–1647.
22. Wang Q, Greene MI. Egr enhances survivin expression through the phosphoinositide 3 (Pi-3) kinase signaling pathway. *Exp Mol Pathol*. 2005;79:100–107.
23. Thorarinsdottir HK, Santi M, McCarter R, et al. Protein expression of platelet-derived growth factor receptor correlates with malignant histology and Pten with survival in childhood gliomas. *Clin Cancer Res*. 2008;14:3386–3394.
24. Rosenbaum C, Kluwe L, Mautner VF, Friedrich RE, Muller HW, Hanemann CO. Isolation and characterization of Schwann cells from neurofibromatosis Type 2 patients. *Neurobiol Dis*. 1998;5:55–64.
25. Utermark T, Kaempchen K, Antoniadis G, Hanemann CO. Reduced apoptosis rates in human schwannomas. *Brain Pathol*. 2005;15:17–22.
26. Hanemann CO, Bartelt-Kirbach B, Diebold R, Kampchen K, Langmesser S, Utermark T. Differential gene expression between human schwannoma and control Schwann cells. *Neuropathol Appl Neurobiol*. 2006;32:605–614.
27. Lallemand D, Manent J, Couvelard A, et al. Merlin regulates transmembrane receptor accumulation and signaling at the plasma membrane in primary mouse Schwann cells and in human schwannomas. *Oncogene*. 2009;28:854–865.
28. Hafizi S, Dahlback B. Signalling and functional diversity within the Axl subfamily of receptor tyrosine kinases. *Cytokine Growth Factor Rev*. 2006;17:295–304.
29. Camp ER, Liu W, Fan F, Yang A, Somcio R, Ellis LM. Ron, a tyrosine kinase receptor involved in tumor progression and metastasis. *Ann Surg Oncol*. 2005;12:273–281.
30. Wang MH, Lee W, Luo YL, Weis MT, Yao HP. Altered expression of the Ron receptor tyrosine kinase in various epithelial cancers and its contribution to tumorigenic phenotypes in thyroid cancer cells. *J Pathol*. 2007;213:402–411.
31. Wang J, Rajput A, Kan JL, et al. Knockdown of Ron kinase inhibits mutant phosphatidylinositol 3-kinase and reduces metastasis in human colon carcinoma. *J Biol Chem*. 2009;284:10912–10922.
32. Angeloni D, Danilkovitch-Miagkova A, Miagkov A, Leonard EJ, Lerman MI. The soluble sema domain of the Ron receptor inhibits macrophage-stimulating protein-induced receptor activation. *J Biol Chem*. 2004;279:3726–3732.
33. Hilton DA, Ristic N, Hanemann CO. Activation of Erk, Akt and Jnk signalling pathways in human schwannomas in situ. *Histopathology*. 2009;55:744–749.
34. James MF, Han S, Polizzano C, et al. Nf2/Merlin is a novel negative regulator of Mtor complex 1, and activation of Mtorc1 is associated with meningioma and schwannoma growth. *Mol Cell Biol*. 2009;29:4250–4261.
35. Lopez-Lago MA, Okada T, Murillo MM, Socci N, Giaccotti FG. Loss of the tumor suppressor Gene Nf2, encoding Merlin, constitutively activates integrin-dependent Mtorc1 signaling. *Mol Cell Biol*. 2009;29:4235–4249.
36. Doherty JK, Ongkeko W, Crawley B, Andalibi A, Ryan AF. ErbB and Nrg: potential molecular targets for vestibular schwannoma pharmacotherapy. *Otol Neurotol*. 2008;29:50–57.
37. Birchmeier C, Nave KA. Neuregulin-1, a key axonal signal that drives Schwann cell growth and differentiation. *Glia*. 2008;56:1491–1497.
38. Hansen MR, Linthicum FH Jr. Expression of neuregulin and activation of ErbB receptors in vestibular schwannomas: possible autocrine loop stimulation. *Otol Neurotol*. 2004;25:155–159.
39. Montemurro F, Valabrega G, Aglietta M. Lapatinib: a dual inhibitor of Egr and Her2 tyrosine kinase activity. *Expert Opin Biol Ther*. 2007;7:257–268.
40. Xia W, Mullin RJ, Keith BR, et al. Anti-tumor activity of Gw572016, a dual tyrosine kinase inhibitor blocks Egr activation of Egr/ErbB2 and downstream Erk1/2 and Akt pathways. *Oncogene*. 2002;21:6255–6263.
41. Rusnak DW, Affleck K, Cockerill SG, et al. The characterization of novel, dual ErbB-2/Egr, tyrosine kinase inhibitors: potential therapy for cancer. *Cancer Res*. 2001;61:7196–7203.
42. Rusnak DW, Lackey K, Affleck K, et al. The effects of the novel, reversible epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor, Gw2016, on the growth of human normal and tumor-derived cell lines in vitro and in vivo. *Mol Cancer Ther*. 2001;1:85–94.
43. Spector NL, Xia W, Burris H 3rd, et al. Study of the biologic effects of lapatinib, a reversible inhibitor of ErbB1 and ErbB2 tyrosine kinases, on tumor growth and survival pathways in patients with advanced malignancies. *J Clin Oncol*. 2005;23:2502–2512.
44. Hassounah M, Lach B, Allam A, et al. Benign tumors from the human nervous system express high levels of survivin and are resistant to spontaneous and radiation-induced apoptosis. *J Neurooncol*. 2005;72:203–208.
45. Katoh M, Wilms R, Belkouch MC, de Tribolet N, Pizzolato G, Dietrich PY. Survivin in brain tumors: an attractive target for immunotherapy. *J Neurooncol*. 2003;64:71–76.
46. Altieri DC. Survivin, cancer networks and pathway-directed drug discovery. *Nat Rev Cancer*. 2008;8:61–70.
47. Gril B, Palmieri D, Bronder JL, et al. Effect of lapatinib on the outgrowth of metastatic breast cancer cells to the brain. *J Natl Cancer Inst*. 2008;100:1092–1103.
48. Lin NU, Carey LA, Liu MC, et al. Phase II trial of lapatinib for brain metastases in patients with human epidermal growth factor receptor 2-positive breast cancer. *J Clin Oncol*. 2008;26:1993–1999.
49. Dhillon S, Wagstaff AJ. Lapatinib. *Drugs*. 2007;67:2101–2108; discussion 2109–2110.