Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Plotkin SR, Stemmer-Rachamimov AO, Barker FG II, et al. Hearing improvement after bevacizumab in patients with neurofibromatosis type 2. N Engl J Med 2009;361:358-67. DOI: 10.1056/NEJMoa0902579.

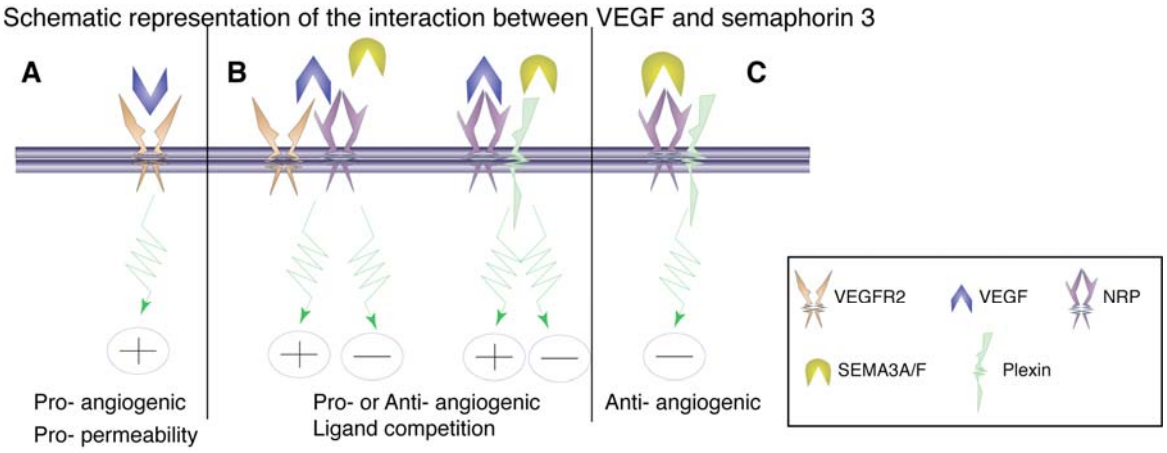
Immunohistochemical analyses

We determined the expression pattern of growth factors associated with tumor angiogenesis in paraffin-embedded tissues of 21 NF2-related schwannomas and 22 sporadic schwannomas. For control specimens, 9 normal spinal nerve roots were obtained from a single autopsy case. Five micron-thick sections were cut and immunostained with the following antibodies: CD31 (Dako, prediluted), αSMA (Sigma, 1:100), VEGF (Santa Cruz or Neomarker 1:100), VEGFR2, PDGFR-α, PDGFR-β (Cell Signaling; 1:250, 1:100 and 1:100 respectively), Neuropilin-1 (Chemicon, 1:40), Neuropilin-2 (R&D Biosystem, 1:500), Semaphorin 3A (Millipore, 1:100) and Semaphorin 3F (Chemicon 1:250). Semiquantitative analysis was performed by two authors who scored the intensity of staining of tumor cells and blood vessels on a scale from 0 (no staining) to 3 (strong staining). For calculation of microvascular density and diameter, CD31 labeling was used to highlight vessels. The quantification used at least 5 fields of confirmed tumor tissue at 200x magnification with an average of 100 vessels counted per section. A customized software analysis tool compatible with Image J (http://rsb.info.nih.gov/ij/) was then used to determine the number of vessels, perimeter, the minor axis of best fitted ellipse (representative of the vessel diameter), and the total surface covered by vascular spaces. The same method was used substituting either VEGFR2 or NRP2 labeling to determine the percentage of vessels expressing these VEGF receptors. Consecutive sections were used to allow similar areas of the tumor to be quantified in all cases.

Hearing evaluations

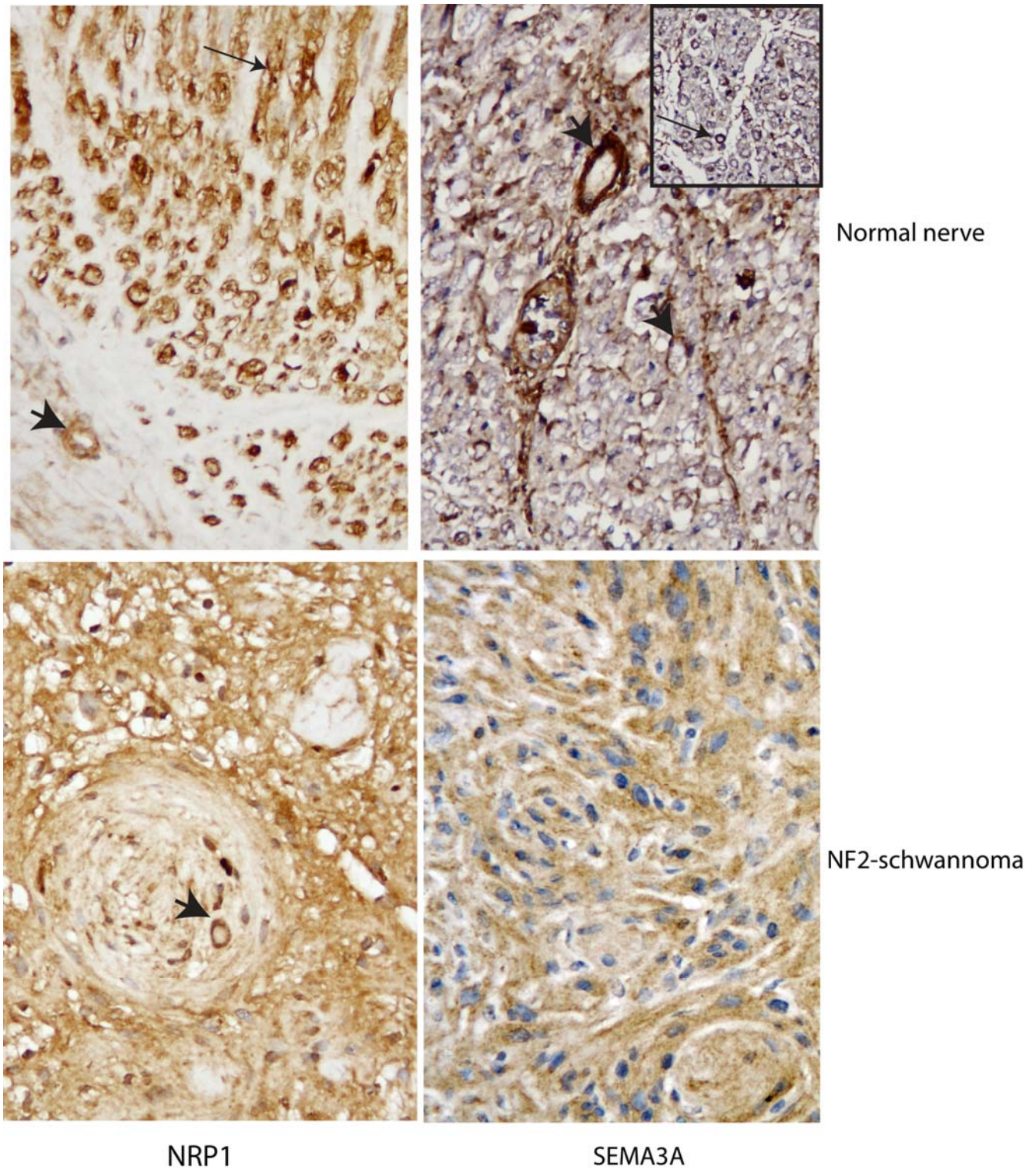
Thresholds were determined at frequencies of 250, 500, 1000, 2000, 3000, 4000, and 8000 Hz and pure tone average (PTA) was calculated as the average of thresholds at 500, 1000, 2000 and 3000 Hz. Word recognition was tested using CID-W22 (Central Institute for the Deaf) monosyllable wordlists.

Supplementary Figures

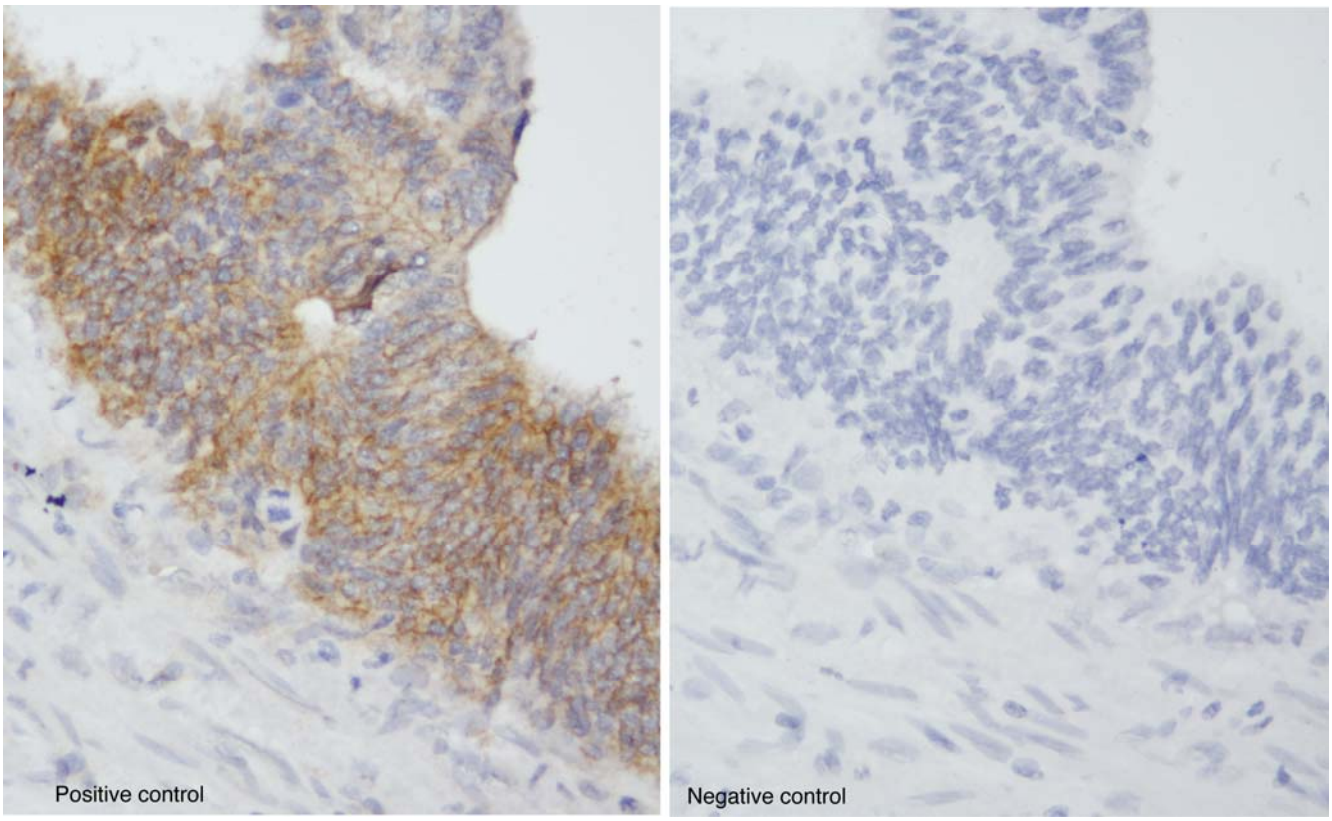


Supplementary Figure 1: Interaction between SEMA3 and VEGF. VEGF typically binds to VEGFR2, inducing endothelial proliferation, migration, and permeability (Panel A). VEGF can also compete with SEMA3 to bind to neuropilin (NRP) receptors directly or can form a bridge between VEGFR2 and NRP potentiating its signaling through VEGFR2 (Panel B). SEMA3A and SEMAF bind NRP1 and NRP2, respectively, to induce endothelial cell *regression* (Panel C). VEGF and SEMA3F have similar affinity for NRP2.

A



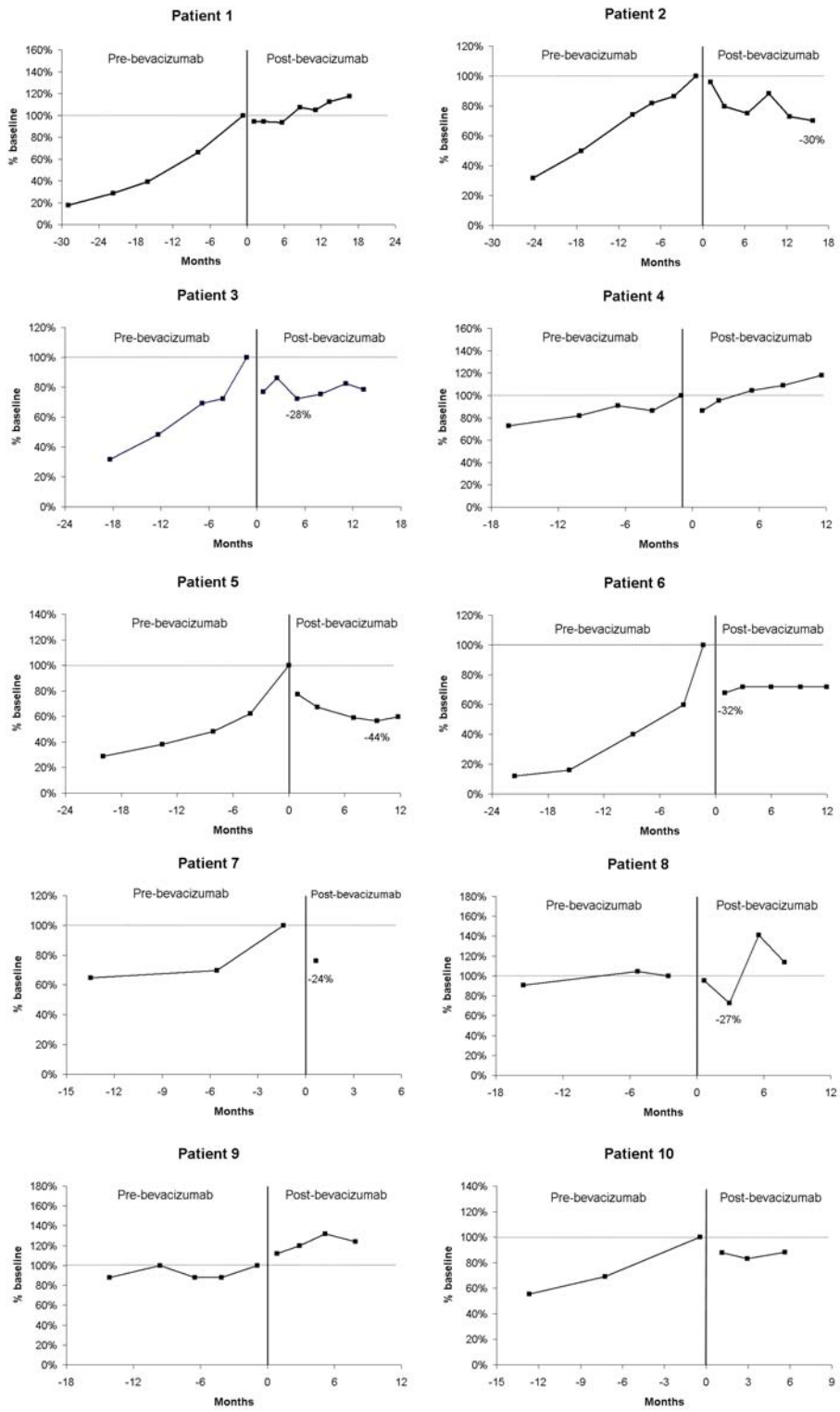
B



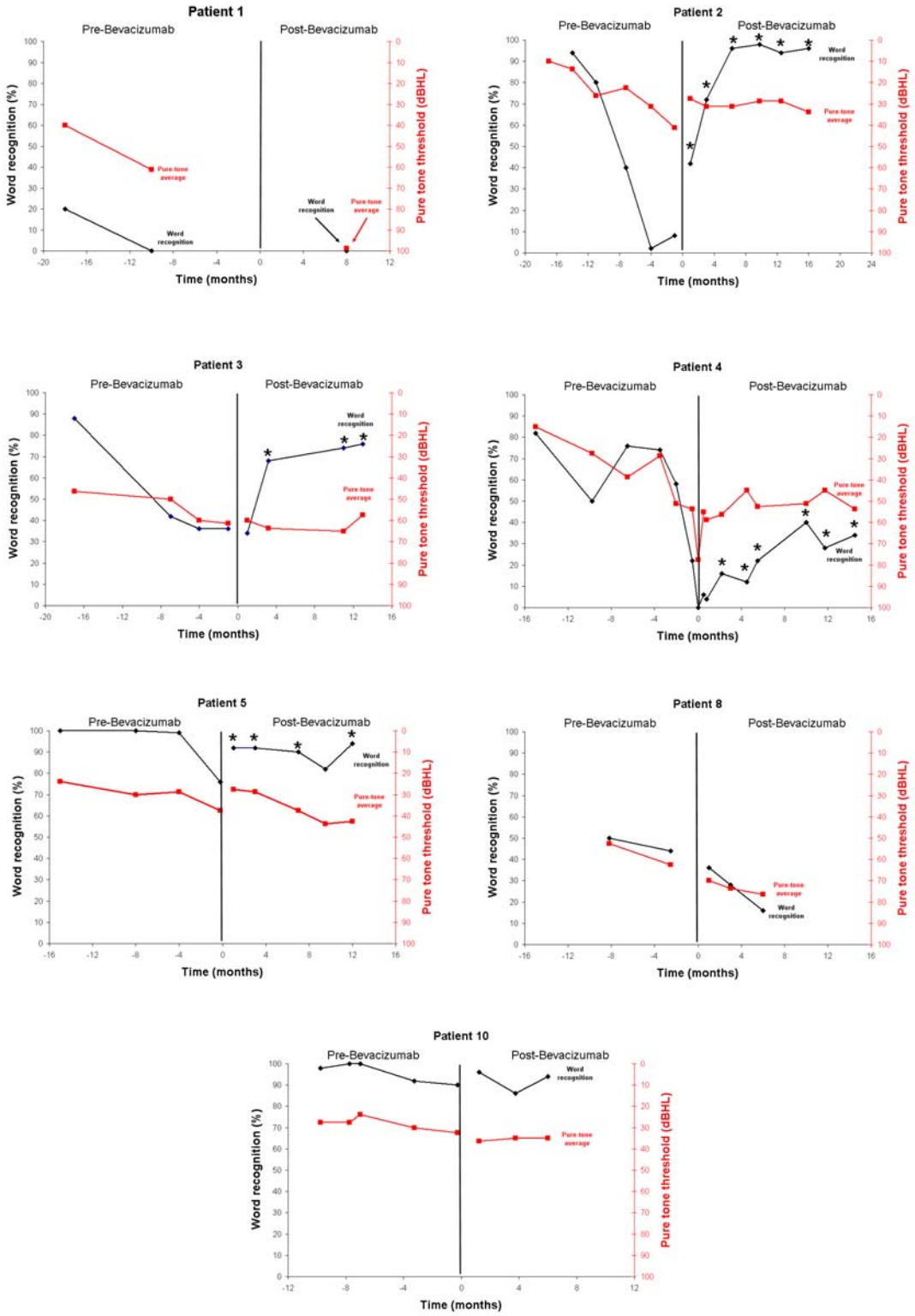
Supplementary figure 2A: Expression pattern of NRP1 and its ligand SEMA3A in normal nerve and schwannomas. Arrowheads point to vessel positive for the given antigen (brown color) while arrows identify Schwann cells. Normal nerve shows strong expression for NRP1 in both vascular endothelium and Schwann cells. In tumors, vessels and tumor cells display positivity. SEMA3A is strongly associated with blood vessels in the normal nerve and can be seen on Schwann cells. The example of tumor shown was chosen amongst the few positive cases in order to illustrate the pattern of expression when detected. For both normal nerves and schwannomas, back-to-back sections were used in order to quantify ligands and receptors in similar regions of the tissues.**2B:** Back-to-back sections were used for positive and negative controls in all IHC experiments in order to assess for the specificity of each antibody. Example shown in this figure is for NRP1.

			NF2		Sporadic				NF2		Sporadic
PDGFRalpha											
	Tumor	0	12		11		Vessels	no	8		8
		1	1		6			yes	6	P<0.05	10
		2	1		1						
PDGFRbeta											
	Tumor	0	9		2		Vessels	0	9		3
		1	7	P<0.05	14			1	0	P<0.001	4
VEGF		2	3		2			2	10		11
	Tumor	0	2		0		Vessels	no	5		10
		1	8		7			yes	15	P<0.05	8
VEGFR2		2	10		11						
	Tumor	0	12		6						
		1	5		8		Vessels		7.4v/mm²		10.4v/mm²
		2	0	P<0.001	3						
Semaphorin 3A		3	0		0						
	Tumor	0	4		12		Vessels	0	1		8
		1	7		6			1	7		7
		2	7	P<0.001	0			2	5		3
Neuropilin 1		3	0		0			3	3		0
								4	2		0
	Tumor	0	2		4		Vessels	0	2		3
		1	4		6			1	2		5
		2	8		6			2	5		1
Semaphorin 3F		3	3		1			3	3		4
								4	5		4
	Tumor	0	12		8		Vessels	0	12		5
		1	5		9			1	5		7
		2	2		0			2	0		2
Neuropilin 2		3	0		0			3	1		2
								4	1		1
	Tumor	0	14		13						
		1	5		5		Vessels		3.3v/mm²		2.3v/mm²
		2	0		0						

Supplementary figure 3: RxC contingency table analysis was performed in order to assess the differences between ordinal variables and to determine statistical differences between NF2-related and sporadic schwannomas. Statistical significance was defined as $P < 0.05$. Only significant comparison are noted in the table. In most cases NF2 and sporadic schwannomas displayed similar distributions in labeling for a given epitope. For tumor cells, scores of 0 to 3 were used to evaluate staining intensity; for tumor vessels, scores of 0 to 4 were used to evaluate the frequency of positive vessels. A score of 0 indicated no vessels positive for a given epitope, a score of 1 indicated rare vessels (less than 5), a score of 2 indicated a few vessels, a score of 3 indicated many vessels (around 50%), and score of 4 indicated most vessels. All quantification was performed by two independent investigators who evaluated each epitope in a single session. The number of tumors used for each epitope varied since some tumor sections were uninterpretable.



Supplemental figure 4. Changes in tumor volume after treatment using bevacizumab 5 mg/kg IV every two weeks. The vertical line indicates the start of treatment (day 0). Radiographic response was defined as a decrease in tumor volume of greater than or equal to 20% compared to the baseline volume; progressive disease was defined as a increase in tumor volume of greater than or equal to 20% compared to the baseline volume; all other responses were defined as stable disease.



Supplementary figure 5. Change in word recognition before and during treatment using bevacizumab for patients eligible for hearing improvement. Patients with normal hearing at initiation of therapy (n=2) and those with surgical resection of both auditory nerves (n=1) were excluded from analysis. Deterioration is expected in word recognition scores over time and even a single case of significant improvement is not expected. Four out of seven treated individuals had an increase in word recognition scores outside the 95% critical threshold. * indicate timepoints qualifying as a hearing response.