Table S1

Immunohistochemical staining for NRP-1

Case	Histology	WHO grade	NRP-1 immunoactivity
J140	Normal	N	-
J141	Normal	N	-
J142	Normal	N	-
J143	Normal	N	-
J40	Pilocytic astrocytoma	I	±
J42	Pilocytic astrocytoma	I	±
J49	Pilocytic astrocytoma	I	±
J51	Pilocytic astrocytoma	I	±
HF1155	Diffuse astrocytoma	II	1+
HF1210	Diffuse astrocytoma	II	2+
HF1367	Diffuse astrocytoma	II	2+
HF1388	Diffuse astrocytoma	II	2+
J12	Diffuse astrocytoma	II	2+
J13	Diffuse astrocytoma	II	1+
J14	Diffuse astrocytoma	II	2+
J44	Diffuse astrocytoma	II	1+
J57	Diffuse astrocytoma	II	1+
J63	Diffuse astrocytoma	II	1+
J50	Oligoastrocytoma	II	1+
HF1016	Oligodendroglioma	II	2+
HF1047	Oligodendroglioma	II	1+
HF1049	Oligodendroglioma	II	±
HF1059	Oligodendroglioma	II	1+
HF1112	Oligodendroglioma	II	-
HF1113	Oligodendroglioma	II	±
HF1156	Oligodendroglioma	II	-
HF1162	Oligodendroglioma	II	1+
HF1167	Oligodendroglioma	II	±
J19	Oligodendroglioma	II	±
J48	Oligodendroglioma	II	1+
J90	Oligodendroglioma	II	1+
J91	Oligodendroglioma	II	2+
HF1032	Anaplastic astrocytoma	III	1+
HF1080	Anaplastic astrocytoma	III	2+
HF1139	Anaplastic astrocytoma	III	1+
HF1295	Anaplastic astrocytoma	III	2+
HF1368	Anaplastic astrocytoma	III	1+
HF1407	Anaplastic astrocytoma	III	2+
J8	Anaplastic astrocytoma	III	2+
J9	Anaplastic astrocytoma	III	2+
J10	Anaplastic astrocytoma	III	2+
J11	Anaplastic astrocytoma	III	1+
J46	Anaplastic astrocytoma	III	1+
J60	Anaplastic astrocytoma	III	2+
J62	Anaplastic astrocytoma	III	±
J15	Anaplastic oligodendroglioma	III	2+
J16	Anaplastic oligodendroglioma	III	2+
J17	Anaplastic oligodendroglioma	III	1+
J18	Anaplastic oligodendroglioma	III	2+
J93	Anaplastic oligodendroglioma	III	3+
J95	Anaplastic oligodendroglioma	III	1+
J96	Anaplastic oligodendroglioma	III	1+
J97	Anaplastic oligoastocytoma	III	2+
HF1025	Glioblastoma multiforme	IV	1+
HF1058	Glioblastoma multiforme	IV	2+
HF1069	Glioblastoma multiforme	IV	3+
HF1071	Glioblastoma multiforme	IV	3+
HF1085	Glioblastoma multiforme	IV	3+
HF1087	Glioblastoma multiforme	IV	1+
HF1096	Glioblastoma multiforme	IV	2+
HF1122	Glioblastoma multiforme	IV	3+
HF1161	Glioblastoma multiforme	IV	1+
J1	Glioblastoma multiforme	IV	3+
J2	Glioblastoma multiforme	IV	2+
J5	Glioblastoma multiforme	IV	2+
J6	Glioblastoma multiforme	IV	2+
J7	Glioblastoma multiforme	IV	2+
J53	Glioblastoma multiforme	IV	2+
J55	Glioblastoma multiforme	IV	2+
J56	Glioblastoma multiforme	IV	1+
J58	Glioblastoma multiforme	IV	1+
J59	Glioblastoma multiforme	IV	1+
J64	Glioblastoma multiforme	IV	±
J65	Glioblastoma multiforme	IV	1+
J66	Glioblastoma multiforme	IV	1+
J67	Glioblastoma multiforme	IV	1+
J68	Glioblastoma multiforme	IV	1+
J70	Glioblastoma multiforme	IV	±
J72	Glioblastoma multiforme	IV	±
J74	Glioblastoma multiforme	IV	1+
J75	Glioblastoma multiforme	IV	2+
J76	Glioblastoma multiforme	IV	2+
J77	Glioblastoma multiforme	IV	2+
J79	Glioblastoma multiforme	IV	1+
J80	Glioblastoma multiforme	IV	1+
J81	Glioblastoma multiforme	IV	2+
J82	Glioblastoma multiforme	IV	2+
J83	Glioblastoma multiforme	IV	2+
J84	Glioblastoma multiforme	IV	2+
J85	Glioblastoma multiforme	IV	1+
J86	Glioblastoma multiforme	IV	1+
J87	Glioblastoma multiforme	IV	2+
J88	Glioblastoma multiforme	IV	3+
J89	Glioblastoma multiforme	IV	2+
J94	Glioblastoma multiforme	IV	1+
J98	Glioblastoma multiforme	IV	2+

Supplementary Material:

Neuropilin-1 Promotes Human Glioma Progression through Potentiating the Activity of HGF Autocrine Pathways

B Hu[1,3,*], P Guo[1,2], I Bar-Joseph[1,2], Y. Imanishi[1,2], MJ Jarzynka[1,2], O Bogler[4], T Mikkelsen[5] T Hirose[6], R Nishikawa[7] and SY Cheng[1,2,*]

University of Pittsburgh [1]Cancer Institute & Department of [2]Pathology or [3]Medicine, Research Pavilion at Hillman Cancer Center, Room 2.19, 5117 Centre Avenue; Pittsburgh, PA 15213; [4]Department of Neurosurgery & Brain Tumor Center, M.D. Anderson Cancer Center, University of Texas, 1515 Holcombe Blvd, Unit 64, Houston, TX 77030; Hermelin Brain Tumor Center, Henry Ford Hospital, 2799 West Grant Blvd, Detroit, MI 48202; Department of [6]Pathology and [7]Neurosurgery, Saitama Medical University, Saitama, 350-0495, Japan

The abbreviations used are: NRP1, neuropilin-1; semaphorin 3A, Sema 3A; HGF/SF, hepatocyte growth factor/scatter factor; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; VEGF-R, VEGF receptor; Akt, v-Akt murine thymoma viral oncogene homolog; Bad, Bcl-2 antagonist of cell death; PTEN, phosphatase and tensin homologue; CM, conditioned media; MVD, microvascular density; IHC, Immunohistochemistry; NRP1 or LacZ, U87MG cells that stably overexpress NRP1 or LacZ, respectively.

Results

NRP1 expression significantly correlates with the malignancy of human gliomas.

We determined whether there is a link between up-regulation of NRP1 and human glioma progression using IHC analysis of 92 primary glioma specimens and 4 normal brain biopsies. As shown in Figure 3B and Table S1, no or weak immunoactivity for the anti-NRP1 antibodies were found in the 4 normal brain tissues (-) and the 4 WHO grade I gliomas (P.A., ±). In WHO grade II and III glioma specimens, 29.2 % of the grade II gliomas (7 out of 24) and 47.6% of the grade III gliomas (10 out of 21) were strongly positive for NRP1 expression (≥2+) whereas 45.8 % of grade II (11 out 24) and 47.6 % of grade III tumors (10 out of 21) expressed NRP1 at moderate levels (1+). In a total of 43 WHO grade IV glioma specimens examined, 55.8% (24 out of 43 biopsies) tumors showed high levels of NRP1 expression (2+ or 3+) and 37.2% tumors expressed NRP1 at moderate levels (16 out of 43). We then performed a Kruskal-Wallis test on these data between normal and grade I tumors versus grade III and IV and found a significant difference ($p<0.0001$). The difference between each group was then examined by a Scheffe's post-hoc test and statistical significance was found again among these groups (Figure 1B). Together, these data show that there is a significant difference in IHC staining for NRP1 among the 3 groups as well as a correlation between NRP1 expression and the malignancy of human glioma.

Materials and Methods

Antibodies and Reagents.

The following reagents were used in this study: a rabbit anti-NRP1 antibody, NP1ECD1A, 1: 100 for IHC (Guo et al., 2001); goat polyclonal anti-NRP1 antibodies, C-19 and H-286, 1:100 dilution for immunohistochemical (IHC) staining and 1:500 dilution for immunoblot (IB) analyses, rabbit anti-c-Met (C-12), mouse anti-Bad (C-17) and goat anti-Sema 3A antibodies

(IB), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); a rat monoclonal anti-mouse CD31 antibody (MEC 13.3), 1:1000 for IHC, and its isotype control $IgG_{2a}.\kappa$, BD PharMingen, Inc. (San Diego, CA); a rabbit polyclonal anti-von Willebrand Factor (vWF) antibody, 1:1000 dilution for IHC, DAKO (Carpinteria, CA); a mouse monoclonal anti-BrdUrd antibody (ready to use), Amersham (Piscataway, NJ); a rabbit anti-ERK1/2 antibody and a phospho-specific polyclonal antibody against activated ERK1/2 (E10 to pT202/pY204), a rabbit anti-p-Bad (Ser-112) antibody and a rabbit anti-p-Bad (Ser-136) antibody, Cell Signaling (Danvers, MA); and a polyclonal anti-Met ($pY^{1230/1234/1235}$) antibody, Biosource/Invitrogen (Carlsbad, CA). The secondary and tertiary antibodies were from Vector Laboratories (Burlingame, CA), Jackson ImmunoResearch Laboratories (West Grove, PA) and Molecular Probes/Invitrogen (Carlsbad, CA). A 3,3'-diaminobenzidine elite kit was from Dako Co. (Carpinteria, CA). Aquablock was from East Coast Biologicals, Inc., (North Berwick, ME). U0126 was from CalBiochem. (San Diego, CA). All other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO), Invitrogen/Life Sciences (Carlsbad, CA) and Fisher Scientific (Hanover Park, IL).

Primary human glioma specimens, IHC and statistical analyses. Of the 96 clinical tissue specimens that were investigated, there were 4 pilocytic astrocytomas (P.A., WHO grade I), 10 diffuse astrocytomas (D.A., WHO grade II), 1 oligoastrocytoma (O.A., WHO grade II), 13 oligodendrogliomas (O.D., WHO grade II), 13 anaplastic astrocytomas (A.A., WHO grade III), 7 anaplastic oligodendrogliomas (A.O.D., WHO grade III), 1 anaplastic oligoastrocytoma (A.O.A., WHO grade III) and 43 glioblastoma multiforme (GBM, WHO grade IV). Four normal human brain biopsies were also included as normal controls. The tumor samples were surgical specimens obtained at the Department of Neurosurgery, Saitama Medical School, Saitama, Japan

and the Department of Neurosurgery, Henry Ford Hospital, Detroit, MI, USA. The normal brain samples were from autopsied patients who did not have any brain lesions. Analyses of histopathology of the clinical specimens, IHC staining and statistics were performed using three well-characterized anti-NRP1 antibodies (Ding et al., 2000) and isotype matched IgGs as negative controls that all showed no staining as previously described (Guo et al., 2005). The specificity of each of these antibodies for detecting NRP1 expression was verified by IB analyses on U87MG or COS-7 cells that were transfected by human NRP1 cDNA (Cackowski et al., 2004). All three anti-NRP1 antibodies produced similar NRP1 expression profiles for IHC staining on these primary glioma specimens when using DAB as a chromogen. The intensities of IHC staining were defined as no stain (-), the reaction was indistinguishable from the background; weakest (±), 5 to 10% of tumor cells were stained; low (1+), 10% to 25% of tumor cells were positive; medium, (2+), 25% to 40 % of tumor cells were positive; and high (3+), more than 40% of tumor cells were positively stained.

Human glioma xenograft models, *in vivo* BrdUrd labeling, IHC and Quantitative Data Analyses. Subcutaneous and intracerebral tumor implantation, measurements of U87MG glioma growth kinetics, preparation of frozen and paraffin-embedded tissue samples, BrdUrd incorporation, IHC and immunofluorescent staining and statistical analyses were performed as previously described (Guo et al., 2003b; Guo et al., 2001).

Immunoprecipitation (IP) and immunoblot (IB) Analyses. Cells and tumor tissues were lysed on ice with a lysis buffer containing 20 mM Tris-HCl (pH 7.0), 100 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF, 10 mM $Na_4P_2O_7$, 0.5% sodium deoxycholate, 1% Triton X-

100, 10% glycerol and protease inhibitor cocktail. Total lysates of various cells or tissues were then subjected to IP analyses using 1 mg of total protein or IB analyses using 30 μg of total protein as previously described except the indicated antibodies against Bad, c-Met or NRP1 were used (Hu et al., 2003). The IB analyses of frozen primary human glioma tissues were performed using anti-NRP1, antic-Met or anti-phosphorylated-c-Met antibodies as previously described (Nishikawa et al., 1998).

Generation of U87MG cell lines that stably express NRP1 or LacZ. Full-length NRP1 cDNA was assembled, validated by sequencing and cloned into a retroviral vector, pLNCX2 as previously described (Cackowski et al., 2004). U87MG cell lines that stably express NRP1 or LacZ encoded by the pLNCX2 vector were generated as previously reported (Nishikawa et al., 1994).

Cell Survival and Proliferation Assays. U87MG parental and NRP1-expressing cells were seeded and cultured in 6-well plates in triplicate in serum-free DMEM at 37^{o}C in 5% CO_2. At 24 hr intervals, cells were trypsinized, stained with trypan blue dye and counted. For neutralizing antibody blocking, the indicated antibodies were included in the serum-free medium for 48 hr. For siRNA inhibition, cells were transiently transfected with gene-specific or control siRNA for 24 hr and then cultured in serum-containing medium for an addition 48 hr.

NRP1-enhanced glioma cell proliferation was assessed as previously described except various concentrations of rhHGF were used in the assays (Guo et al., 2003a). In the experiments for siRNA inhibition of NRP1, the cells were seeded in 96-well plates 48 hr after siRNA transfection.

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