Clinical Cancer Research

Gene Expression Analysis Identifies Potential Biomarkers of Neurofibromatosis Type 1 Including Adrenomedullin

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Abstract

Purpose: Plexiform neurofibromas (pNF) are Schwann cell tumors found in a third of individuals with neurofibromatosis type 1 (NF1). pNF can undergo transformation to malignant peripheral nerve sheath tumors (MPNST). There are no identified serum biomarkers of pNF tumor burden or transformation to MPNST. Serum biomarkers would be useful to verify NF1 diagnosis, monitor tumor burden, and/or detect transformation.

Experimental Design: We used microarray gene expression analysis to define 92 genes that encode putative secreted proteins in neurofibroma Schwann cells, neurofibromas, and MPNST. We validated differential expression by quantitative reverse transcription-PCR, Western blotting, and ELISA assays in cell conditioned medium and control and NF1 patient sera.

Results: Of 13 candidate genes evaluated, only adrenomedullin (ADM) was confirmed as differentially expressed and elevated in serum of NF1 patients. ADM protein concentrati on was further elevated in serum of a small sampling of NF1 patients with MPNST. MPNST cell conditioned medium, containing ADM and hepatocyte growth factor, stimulated MPNST migration and endothelial cell proliferation.

Conclusions: Thus, microarray analysis identifies potential serum biomarkers for disease, and ADM is a serum biomarker of NF1. ADM serum levels do not seem to correlate with the presence of pNFs but may be a biomarker of transformation to MPNST. *Clin Cancer Res; 16(20); 5048–57. ©2010 AACR.*

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder affecting 1:3,500 individuals (1). The *NF1* gene, located on chromosome 17, encodes neurofibromin, a GTPase-activating protein that binds and inactivates Ras proteins. *NF1* is classified as a tumor suppressor gene (2). For example, NF1 patients are at increased risk for developing juvenile myelomonocytic leukemia and pilocytic astrocytomas (3, 4). The hallmark of NF1 is the development of Schwann cell precursor/Schwann cell–derived peripheral nerve sheath tumors, the formation of which is correlated with complete loss of *NF1* expression (2, 5). In childhood, these are plexiform neurofibromas (pNF); tumors associated with larger nerves are found in 30% of patients with NF1 (4, 6). pNFs are diffuse tumors associated with larger nerves that can be disfiguring; the only current treatment is surgical resection. Growth of pNF is variable; irregular borders make volumetric calculations difficult to quantify. pNF can undergo malignant transformation to malignant peripheral nerve sheath tumors (MPNST; refs. 6, 7). MPNST are highly aggressive soft tissue sarcomas with poor prognosis (8). The annual incidence of NF1 patients developing MPNST has been estimated at 1.6 per 1,000, with a lifetime risk of 8% to 13% (9). MPNSTs behave aggressively, with a high rate of recurrence and a predilection to metastasize. Outcomes are favorable only when a small and localized tumor can be completely resected (10); adjuvant chemotherapy has no clear role. In the absence of multiple surgical biopsies, there is no reliable method for detecting pNF transformation to MPNST, although positron emission tomography scan can be helpful in some instances (11). Thus, alternative means of determining neurofibroma growth and transformation are needed.

In other oncologic processes, biomarkers play a key role in diagnosis and treatment. For example, α-fetoprotein secreted by germ cell tumors can indicate response to treatment and elevation of prostate-specific antigen is highly correlative with prostate malignancy (12, 13). Several investigators have collected serum from neurofibromatosis patients, but none have tested the relationship of serum proteins with tumor burden (14–16). PCR analysis of mRNA using Schwann cells in culture revealed several

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

The field of neurofibromatosis research is greatly hampered by the absence of markers of tumor burden. Using whole-genome microarray analysis and a secreted protein database, we identified upregulated transcripts in human plexiform neurofibromas (pNF) and MPNSTs. Further investigation revealed that adrenomedullin (ADM) was found in the serum of neurofibromatosis type 1 (NF1) patients at higher levels than controls and was also found at higher levels in the sera of patients with MPNST compared with those with pNF, suggesting that ADM may be a serum biomarker for malignant transformation. If validated in a larger study, serum levels of ADM could serve as an additional tool in the diagnostic repertoire of the physician when attempting to determine if a pNF has undergone malignant transformation.

factors including basic fibroblast growth factor, platelet-derived growth factor, and midkine that are induced by loss of *NF1*. Midkine was also expressed in human neurofibromas, schwannomas, and various nervous system tumors associated with NF1 (17). Midkine and stem cell factor were found to be enriched in serum from NF1 patients, but these factors have not been correlated with tumor burden (9). Another protein, fetal antigen 1, was found in serum of NF1 patients (n = 13) at higher levels than in healthy controls (n = 177; P = 0.037; ref. 18). However, no large-scale study correlating tumor burden with serum markers has yet been carried out.

Because Schwann cells or their precursors are believed to represent the cell of origin of neurofibromas and MPNSTs, Miller et al. (19) compared Schwann cell expression with that of MPNST cell lines and primary MPNSTs and validated the MPNST cell line signature. Schwann cells were also compared with neurofibromas and MPNSTs. Here, we have integrated NF1 transcription data and the human secretome to identify potential serum biomarkers of pNF and/or MPNST. Two candidates [adrenomedullin (ADM) and hepatocyte growth factor (HGF)] emerged from our investigation as being potential biomarkers in patients with NF1.

HGF and its receptor, the tyrosine kinase c-Met, have been implicated in the growth of neoplasms (20) and are implicated in angiogenesis (21). A c-Met/HGF autocrine loop has been implicated in MPNST cell invasion (22). Indeed, HGF and c-Met are expressed by MPNSTs (23) and neurofibromas (23, 24), and HGF is a mitogen for Schwann cells (25).

ADM is a 52–amino acid secreted peptide that belongs to the calcitonin superfamily of peptides based on its structural similarity with calcitonin gene–related peptide and was originally isolated from a pheochromocytoma in 1993 (26). ADM also plays an essential role in growth of pheochromocytomas, neuroblastomas, glioblastomas, and other tumors (27). ADM has been identified as a secreted peptide involved in tumor-induced angiogenesis that results in neovascularization (28, 29) and hypoxia, upregulating ADM in human glioma cell lines as well as promoting glioma growth (30). ADM mRNA is increased in higher-grade gliomas and cell lines compared with normal brain (31). Further evidence of the role of ADM in tumor development is seen in a glioblastoma multiforme xenograft tumor model, in which administration of anti-ADM antibody greatly reduced both tumor growth and vascularity (31). We present data showing that ADM is increased in the serum of NF1 patients with MPNSTs and may affect MPNST biology via directly enhancing cell migration.

Materials and Methods

Microarray generation

Identification of candidate secreted biomarkers. We generated a list of candidate secreted NF1 biomarkers by intersecting genes statistically differentially expressed between cultured normal human Schwann cells (NHSC), Schwann cells derived from neurofibroma, and MPNST cell lines with proteins cataloged in the Secreted Protein Database (32). Specifically, 9,473 transcripts had been identified in a previous study comparing independent cultures of primary human Schwann cells (n = 10), dermal neurofibroma-derived Schwann cell cultures (n = 11), pNF-derived Schwann cell cultures (n = 11), MPNST cell line cultures (n = 13), dermal neurofibromas (n = 13), pNFs (n = 13), and MPNSTs (n = 6; ref. 33). In brief, normalized samples using the Robust Multichip Analysis algorithm as implemented in Bioconductor/R (34) had been referenced by batch to controls for batch-to-batch variation, and the normalized expression of each gene in each sample to the median expression of the measurements of that transcript across 10 primary NHSC cultures referenced. We had corrected results from the primary analysis for multiple testing effects by applying the Benjamini and Hochberg false discovery rate correction (FDR ≤ 0.05; ref. 35). All statistical comparisons and data visualizations were done using GeneSpring GX v7.3.1 (Agilent Technologies). The data underlying this analysis can be accessed in Gene Expression Omnibus (accession number GSE14038 at http://www.ncbi.nlm.nih.gov).

For the current study, we initially focused on genes upregulated 1-fold or greater between NHSCs and primary neurofibroma or NHSCs and primary MPNST; 1,664 transcripts were upregulated in at least one of the two conditions. We separately imported 3,721 proteins from the Secreted Protein Database (32) into GeneSpring GX using RefSeq ID (confidence rank, 0–3). We then identified the intersection of the list of genes upregulated in NF1 cell cultures with the list of putative secreted proteins upregulated in primary tumors; 666 transcripts were identified. Subsequent cluster analysis across solid tumors (neurofibroma and MPNST) compared with NHSCs (33) identified the 132 most significantly upregulated transcripts (P < 0.001), of which 92 were unique genes and considered candidate secreted biomarkers.

Validation with quantitative real-time PCR. We used total RNA as a template to synthesize double-stranded cDNA using an oligo(dT) primer with SuperScript II reverse transcriptase (Invitrogen). We conducted duplicate reactions omitting reverse transcriptase to control for genomic DNA contamination. Relative levels of RNA were measured by quantitative real-time PCR (qPCR) using the ABI 7500 Sequence Detection System default settings. Amplification was conducted in SYBR Green Master Mix (Applied Biosystems). We obtained primer sequences using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and oligonucleotides were purchased from Integrated DNA Technologies. The following primers were used: ADM, 5′-AGTCGTGGGAAGAGGGAACT-3′ (forward) and 5′-CCCTGGAAGTTGTTCATGCT-3′ (reverse); HGF, 5′-CTGGTTCCCCTTCAATAGCA-3′ (forward) and 5′-CTCCAGGGCTGACATTTGAT-3′ (reverse); β-actin, 5′-GGACTTCGAGCAAGAGATGG-3′ (forward) and 5′-ACATCTGCTGGAAGGTGGAC-3′ (reverse). The remainder of the candidate primers are shown in Supplement 1. Cycle threshold values were obtained, where fluorescence intensity was in the geometric phase of amplification, and averaged for triplicate reactions. Values for individual genes of interest were normalized to values for β-actin and used to calculate fold change in gene expression using ABI software.

ADM and HGF detection in serum-free conditioned medium. MPNST cell lines (described in ref. 19) were grown to 90% confluence in DMEM with 10% fetal bovine serum (FBS). Medium was removed and cells were washed three times with PBS. We then added serum-free DMEM (Fisher Scientific) and removed the medium after 48 hours. We centrifuged the supernatant at 10 × *g* for 5 minutes to remove cellular debris.

Migration assay. We measured the migratory response of 8814 MPNST cells using a modified Boyden chamber assay. Cells (4×10^4) in serum-free DMEM were plated on the upper chamber of a 24-well Transwell plate with 8-μm pore membranes (Costar, Corning, Inc.). The lower chamber contained 800 μL of 8814 MPNST conditioned medium. Cells were incubated for 24 hours at 37°C in 10% CO_2. Nonmigrating cells were removed from the upper surface of the membrane with cotton swabs. Membranes were stained with bisbenzimide and mounted onto glass slides. For each condition, migration was quantified by counting cells in four nonoverlapping fields in three independent membranes. Samples were evaluated in three independent experiments, and data from a representative experiment are shown. Numbers of migrated cells were normalized to the total number of cells on an unscraped filter to validate the total number of cells plated. ADM and ADM 22-52 (a competitive receptor antagonist) were from Bachem Bioscience, Inc. Data shown are representative of three independent experiments; values presented are the mean ± SD. We determined statistical significance by Student's *t* test using GraphPad Prism software.

Patient serum. Serum specimens and corresponding clinical data were collected and used in accordance with a Cincinnati Children's Hospital Medical Center (CCHMC) Institutional Review Board (IRB)–approved protocol. All patients from Cincinnati Children's monthly NF1 comprehensive genetics clinic who had documented pNFs were approached to provide a serum sample. We procured blood samples from 32 NF1 patients. Serum was separated within

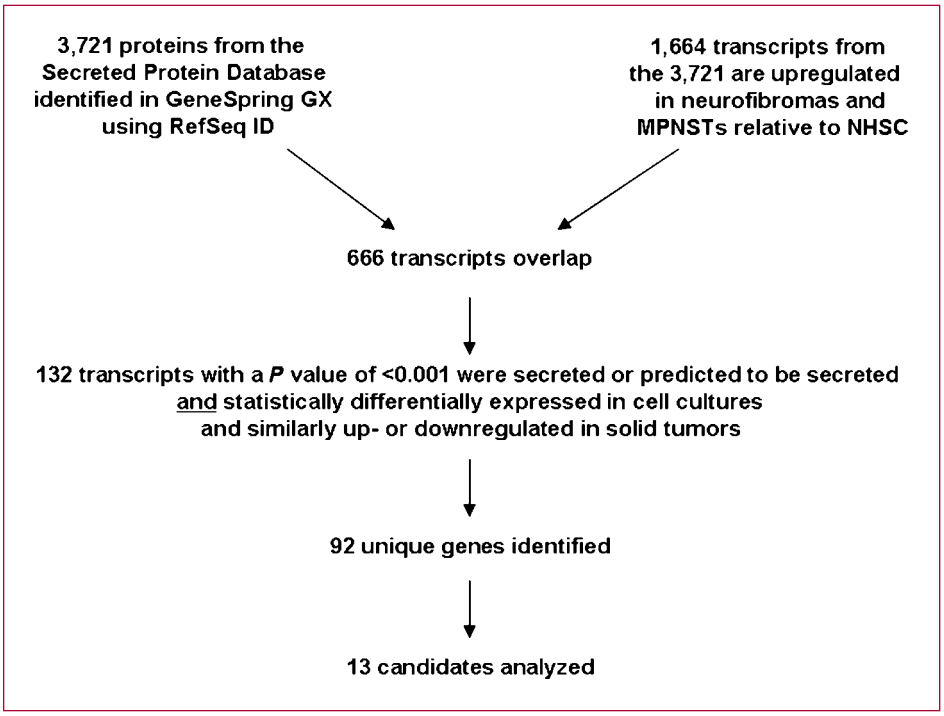

Fig. 1. NF1 biomarker list generation and validation flowchart.

Table 1. List of 13 genes predicted to be overexpressed in NF1 and tested for differential expression

Microarray confirmation	qPCR validation: fold change (range)		Confirmed in MPNST cell conditioned medium		Confirmed in patient serum
	NFSC	MPNST	Western blot	ELISA	
EFEMP1	Failed	Failed	Not done	Not done	Not done
MEGF10	Failed	Failed	Not done	Not done	Not done
KIAA0114	Failed	Failed	Not done	Not done	Not done
CTGF	Failed	Failed	Not done	Not done	Not done
NOS3	Failed	Failed	Not done	Not done	Not done
FA1	Failed	Failed	Not done	Not done	Not done
NPTX2	Failed	159 (7-382)	Failed	Failed	Not done
HSPD1	8 (4-13)	11 (4-24)	Failed	Failed	Not done
IGF1	4 (3-5)	3 (3-4)	Not done	Failed	Not done
MMP9	4 (2-13)	428 (15-2,003)	Not done	Failed	Not done
DAF	6 (2-13)	22 (2-51)	Not done	Failed	Not done
HGF	4 (1-8)	273 (86-917)	Not done	+	Failed
ADM	44 (13-70)	70 (6-193)	Not done	+	+

NOTE: Those candidates that did not fail confirmation have their respective fold change with range in parentheses listed. Quantitative reverse transcription-PCR (Failed = tested in designated experiment and did not confirm at 3-fold or greater change in expression). Data represent mean fold change across cells from four individual patients; range is shown in parentheses. Data represent mean fold change across eight cell lines; range is shown in parentheses. Candidates whose differential expression was confirmed by PCR were investigated for presence in MPNST serum-free medium by Western blot or ELISA (Failed = not detected). ADM and HGF were confirmed in cell conditioned medium and investigated in patient serum via ELISA.

1 hour of collection and kept at −80°C until analyzed. One sample was from an NF1 patient with a pNF who had previously consented to donate serum and subsequently developed an MPNST. We obtained 25 frozen surplus serum samples from the Cincinnati Children's Hospital Clinical Laboratory, which were used as controls. Only age and gender of the surplus serum were revealed in accordance with a CCHMC IRB-approved protocol. Genetics, hematology/oncology, and bone marrow transplant patients were excluded.

ELISA

We conducted ELISA assays on serum-free conditioned medium and human serum using an ADM detection kit (Phoenix Pharmaceuticals) and Human HGF ELISA kit (R&D Systems) following the manufacturer's suggested protocol. We did all assay procedures in triplicate. Internal negative and positive quality controls were provided in each ELISA kit and run in triplicate in each assay.

Tube formation

Human umbilical vascular endothelial cells (HUVEC) were purchased from Lonza. HUVECs maintained in EBM2 medium (Lonza) with supplement (supplied by Lonza) were washed twice with PBS, trypsinized, and plated (4×10^5 cells/mL) in wells of 96-well plates coated with Matrigel (BD Biosciences) in either DMEM or EBM2 with 2% FBS supplemented with ADM (10 μmol/L), ADM 22-52 (10 μmol/L), both ADM and ADM 22-52, HGF (10 ng/mL), or anti-HGF antibody (0.5 μg/mL) in triplicate. After 18 hours, the plates were stained using calcein-AM (8 μg/mL) and two nonoverlapping fields per well were photographed using a fluorescent microscope, accounting for >80% of the area of each filter. Three wells were analyzed per experimental condition. Three independent experiments were conducted, and data from a representative experiment are shown. The area of tube formation was then quantified using ImageJ software.

Results

Biomarker list generation and validation

A flowchart outlining the methodology used to generate a candidate NF1 biomarker list is shown in Fig. 1. We used transcription data collected and analyzed by the NF1 Systems Biology and Microarray Consortium (33) and integrated it with cataloged in the Secreted Protein Database (32). After importing 3,721 RefSeq IDs corresponding to proteins cataloged in the Secreted Protein Database (32), we focused on 1,664 transcripts that were upregulated in cells and primary tumors relative to NHSCs. Of the 1,664 transcripts, 132 were similarly upregulated in both neurofibroma-derived Schwann cell cultures and primary tumors and secreted or predicted to be secreted from the Secreted Protein Database, after imposing a P value of 0.001. After correcting for transcript variants, 92 genes were identified to encode putative secreted proteins and statistically differentially expressed and upregulated in neurofibroma-derived Schwann cell cultures and primary tumors.

Thirteen candidates were chosen for further validation, as they exhibited robust differential expression predicted by the microarray data. Additional criteria for pursuing validation were literature reports of involvement in oncologic processes and/or commercially available reagents. Seven of 13 candidates were validated by qPCR (i.e., differentially expressed in neurofibroma Schwann cells (NFSC) and MPNST versus NHSCs at the mRNA level. Of these seven, two candidates were detected in serum-free conditioned medium and investigated further (Table 1).

ADM and HGF were chosen from the list of 92 genes due to the role of HGF in cell growth and motility and its binding to the proto-oncogenic c-Met receptor (36). ADM was chosen due to its known effects on tumor growth in other cancer models (27). Both candidates exhibited robust expression profiles across all cell lines and primary tumors when compared with NHSCs, and their expression was validated at the RNA level by quantitative qPCR. Figure 2 shows the marked upregulation of ADM and HGF in both MPNST cell lines and neurofibroma-derived primary Schwann cells ($NF1^{+/-}$ and $NF1^{-/-}$) when compared with NHSCs. Intriguingly, there is a decrease in differential expression in the $NF1^{-/-}$ cell strains versus the $NF1^{+/-}$ Schwann cell strains. More samples would be required to determine whether this is a genotype effect or reflects differences among cells from different individuals.

Four of eight tested MPNST cell lines remained viable in serum-free medium for at least 48 hours, enabling collection of conditioned medium. We found that the concentration of ADM in serum-free conditioned medium ranged from 0.152 to 1.072 ng/mL in MPNST cell lines. HGF in MPNST serum-free conditioned medium ranged from 0.426 to 25.01 ng/mL. A sporadic MPNST cell line (STS26T) exhibited the lowest amount of HGF in serum-free conditioned medium at 0.426 ng/mL. This was significant compared with 0.052 ng/mL of HGF in NHSC conditioned medium (Fig. 3A and B). All four tested MPNST cell lines contained ADM and HGF in conditioned medium, suggesting secretion of both HGF and ADM by MPNST cells.

Serum ADM and HGF levels in NF1 patients

Based on ADM and HGF secretion into MPNST conditioned medium, we reasoned that these proteins might be detectable in the serum of patients with NF1 or might be elevated in the serum of patients with significant tumor burden. To test these ideas, we collected serum samples from NF1 patients with and without pNF as well as NF1 patients with MPNST. Thirty-two patients with NF1

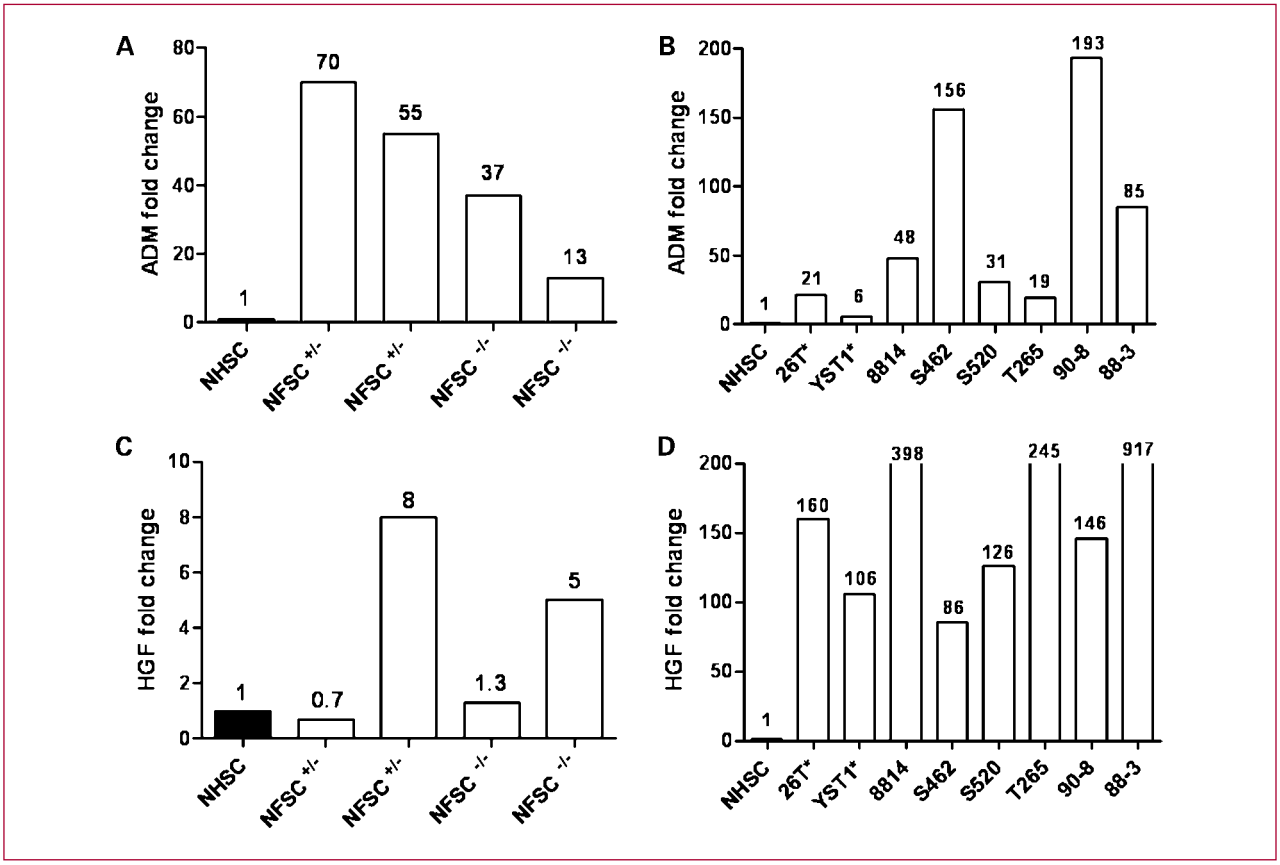

Fig. 2. Quantitative PCR validating increased expression of ADM and HGF mRNAs. ADM differential expression in primary NFSCs (A) and MPNST cell lines (B). Fold change is noted above each bar. *, sporadic MPNST cell lines. $NFSC^{+/-}$, *NF1* heterozygous NFSCs; $NFSC^{-/-}$, *NF1*-null NFSC. HGF differential expression in NFSCs (C) and MPNST cell lines (D). For validation, 3-fold change or greater was considered significant.

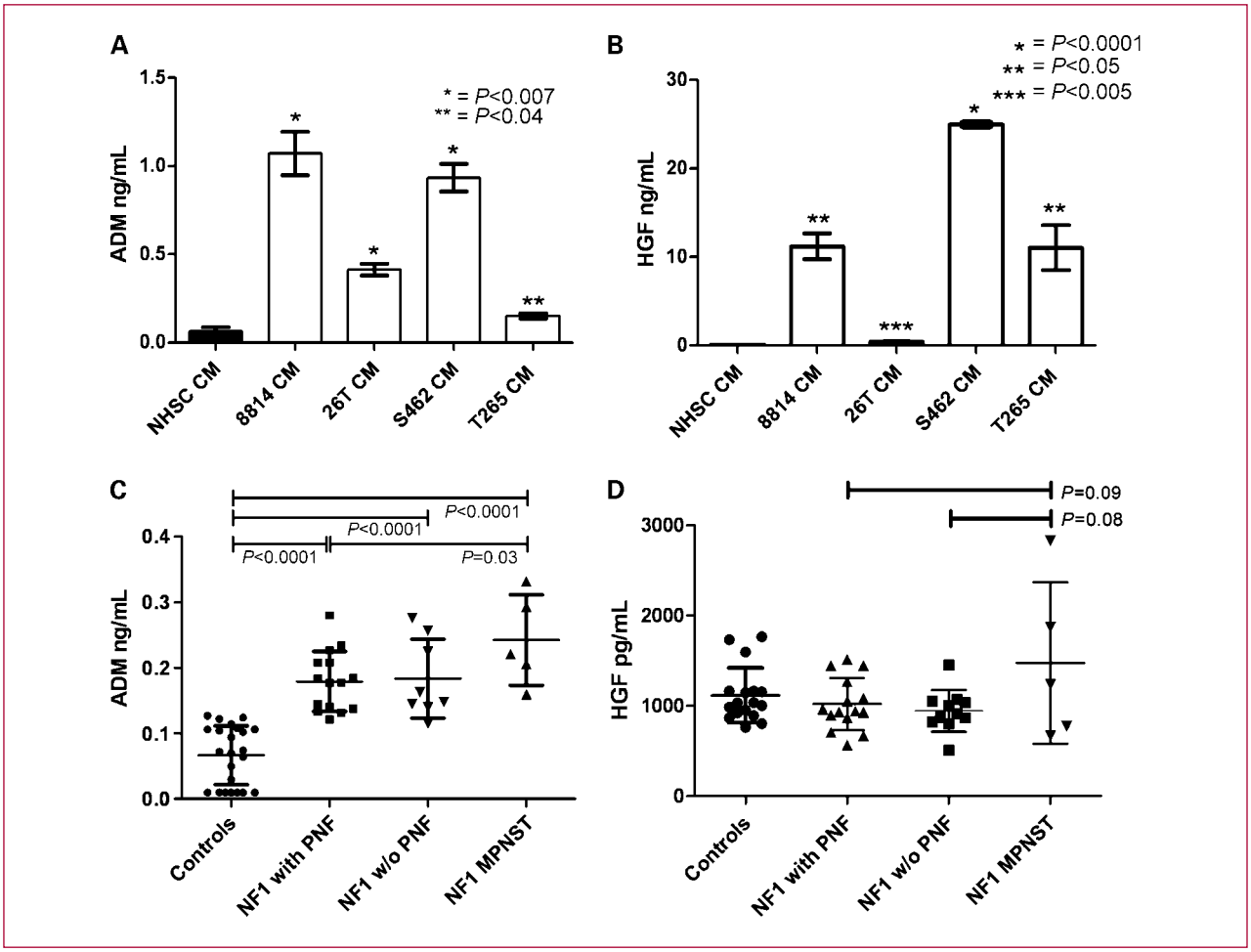

Fig. 3. ELISA assays confirm increased expression of ADM and HGF proteins in cell conditioned medium; ADM is elevated in serum from NF1 patients and may be further elevated in MPNST serum. ADM (A) and HGF (B) levels in MPNST serum-free conditioned medium (CM) are increased compared with NHSC serum-free conditioned medium. 26T is a sporadic, non-NF1 MPNST cell line. 8814, S462, and T265 are NF1 patient–derived cell lines. Student's two-tailed *t* test was used for statistical analyses. C, ELISA assays monitored serum levels of ADM in NF1 patient sera compared with controls. D, ELISA assays monitored HGF in the same samples as in C. There was no difference in the levels of HGF in NF1 patient samples compared with controls. In C, Student's two-tailed *t* test compared controls with each individual sample type, or NF1 patients with pNF to NF1 patients with MPNST. In D, no significant differences were identified by Student's two-tailed *t* test among any two groups.

consented to have blood drawn for analysis. Ten patients did not have pNF, whereas 17 patients had documented pNF, defined by clinical exam and imaging (computed tomography or magnetic resonance imaging). The age and gender distribution of the 32 participating NF1 patients are shown in Table 2. One patient was diagnosed with an MPNST of the abdomen 2 weeks after collection, and the corresponding serum was grouped with the MPNST serum samples.

The mean ADM serum concentration in controls was 0.07 ng/mL ($n = 25$), significantly lower ($P < 0.0001$) than serum ADM levels in NF1 patients with ($n = 17$) or without ($n = 10$) pNF (mean serum ADM concentration of 0.18 ng/mL for both groups; Fig. 3C). The mean ADM serum concentration in controls was also significantly lower than the mean serum concentration for NF1 patients with MPNSTs (mean ADM concentration of 0.24 ng/mL; $P < 0.0001$). There were no differences in mean ADM serum levels when comparing NF1 patients with pNF and NF1 patients without pNF. In addition, there was no difference in ADM serum levels when comparing age, gender, or location of pNF. However, mean ADM concentration in NF1 patients with MPNSTs had significantly higher ADM levels when compared with NF1 patients with pNF (mean ADM concentration of 0.24 versus 0.18 ng/mL; $P = 0.03$). In contrast, HGF was not significantly different in the serum of controls (1.1 ng/mL) compared with NF1 patients with pNF (1.0 ng/mL), NF1 patients without pNF (0.95 ng/mL), or NF1 patients with MPNSTs (1.5 ng/mL; Fig. 3D).

ADM stimulates migration of MPNST cells

ADM is implicated in the biology of neuroblastoma and glioblastoma as well as lung, breast, and ovarian neoplasms (27, 37), in both tumor cell proliferation and migration

(38, 39). To test for potential functions of ADM in MPNST, we conducted cell migration experiments using ADM as a stimulator of migration and ADM 22-52 as a competitive receptor antagonist (40, 41). Stimulation of migration by ADM was tested using an NF1-associated cell line (8814) and a sporadic MPNST cell line (26T; Fig. 4A and B). The migration of 8814 MPNST cells was stimulated 1.5-fold when ADM at 10 μmol/L was added to DMEM with 10% FBS ($P < 0.0001$). Adding ADM 22-52 at 10 μmol/L reduced migration by 1.5-fold ($P = 0.0003$). The combination of both ADM and ADM 22-52 normalized migration to baseline ($P = 0.07$). A more dramatic effect was seen when using serum-free conditioned 8814 MPNST medium and comparing its migration to serum-free DMEM; a 6-fold increase in migration was seen ($P < 0.0001$). A 4-fold increase in migration occurred when using 26T serum-free conditioned medium compared with serum-free DMEM ($P < 0.0001$). The addition of ADM 22-52 to both 8814 and 26T conditioned medium nearly normalized the migration effect to that of serum-free DMEM.

Figure 4B shows that 26T MPNST cells were stimulated 1.3-fold when ADM at 10 μmol/L is added to DMEM with 10% FBS ($P < 0.0001$). Adding ADM 22-52 at 10 μmol/L reduced migration by 1.8-fold ($P < 0.0001$). Compared with the 8814 MPNST data, a lesser effect was seen when using serum-free conditioned 26T MPNST medium and comparing its migration to serum-free DMEM; only a 1.6-fold increase in migration was seen ($P = 0.0012$). No statistically significant increase or decrease in migration was seen when using serum-free conditioned medium with ADM 22-52 or 8814 serum-free conditioned medium ($P = 0.48$).

NF1-associated MPNST serum-free conditioned medium stimulates HUVEC tube formation

HGF and ADM each stimulate proliferation in HUVECs (21, 42, 43), and vascularity may be associated with poorer prognosis in some tumors (44, 45). We did experiments to elucidate whether MPNST serum-free conditioned medium, shown above to contain ADM and HGF, would stimulate endothelial tube formation. As a control, ADM and HGF were added to EGM2 (HUVEC base medium) and shown to induce more proliferation than baseline (data not shown). ADM and HGF inhibitors were also tested in EGM2 and shown to reduce HUVEC tube formation (data not shown). Serum-free conditioned media from 8814 (NF1-associated MPNST) and 26T (sporadic MPNST) were likewise tested on HUVECs. Area of tube formation was then compared with that of HUVECs in serum-free DMEM. The respective MPNST conditioned medium was subsequently combined with ADM and HGF inhibitors; the results of which are shown in Fig. 4C and D.

NF1-associated MPNST serum-free conditioned medium (8814) stimulated more tube formation than the control serum-free DMEM ($P < 0.0001$; Fig. 4C). There was no statistical difference between 8814 serum-free conditioned medium and EGM2 (HUVEC base medium). Antagonists of ADM and HGF prevented tube formation when HUVECs were exposed to 8814 serum-free conditioned medium. Sporadic MPNST serum-free conditioned medium did not stimulate tube formation compared with serum-free DMEM (Fig. 4D).

Table 2. Characteristics of the NF1 patients whose serum was drawn for analysis

	NF1 without pNF	NF1 with pNF	MPNST	Controls
Total	10	17	5	25
NF1	9	17	5	n/a
Sibling control	1	n/a	n/a	n/a
Sex				
Male	7	6	3	15
Female	3	11	2	10
Age (y)				
Range	13-42	5-42	16-50	4-19
Mean	21	19	30	13
Median	17	16	20	15

Abbreviation: n/a, not applicable or unknown.

Discussion

The present study describes integration of NF1 transcriptional data and human proteins known or predicted to be secreted. From a list of 92 putative secreted NF1 biomarkers, we identified two candidates: ADM and HGF. ADM and HGF mRNA differential expression were validated by qPCR. Both proteins are secreted in serum-free conditioned medium from sporadic and NF1-associated MPNST cell lines. We show that ADM is a serum biomarker of NF1; NF1 patients with and without pNF, and with MPNST, have significantly increased serum concentrations of ADM compared with controls. We also show that ADM has cell-autonomous effects on MPNST migration. Whereas 13 candidates were initially investigated, another 79 candidates have yet to be validated. Further investigation of these candidates using serum-free conditioned medium and the sera of patients with pNF and MPNST as described above could lead to additional biomarkers and potential therapeutic targets

Previous investigations have illustrated the benefit of using microarrays to arrive at biomarkers that may predict neoplasm development. Dhanasekaran et al. (46) used microarrays in prostate cancer and were able to delineate *hepsin* and *pim-1* proteins as potential biomarkers in prostate cancer. Other studies have taken further steps, similar to those described in this study, in elucidating potential biomarkers. Mok et al. used microarrays to identify overexpressed genes for secretory proteins and confirmed in patient sera via ELISA that prostasin may be a serum biomarker for ovarian cancer. Prostasin, when studied in combination with the known biomarker CA-125, has a sensitivity of 92% and a specificity of 94% in detecting

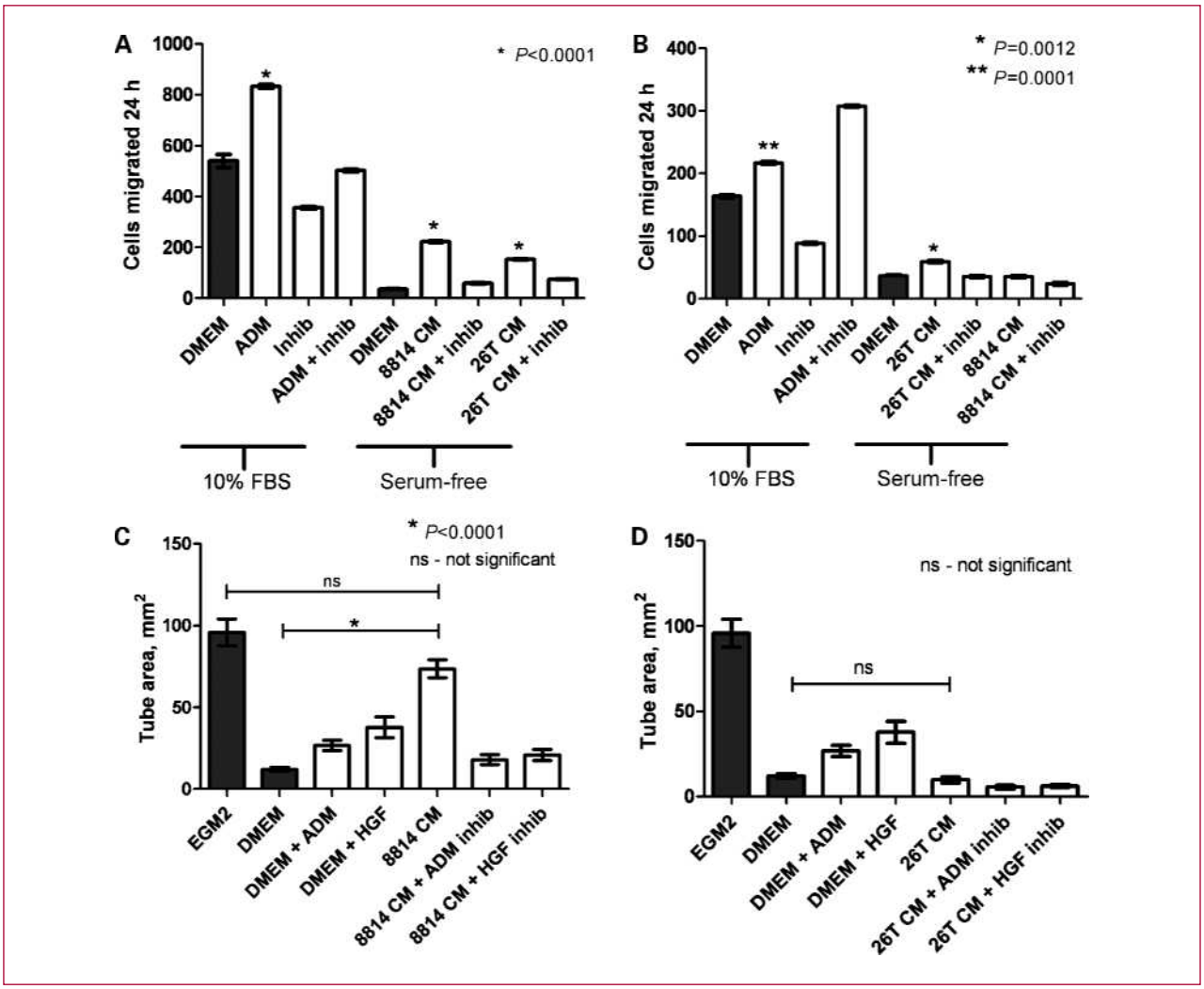

Fig. 4. ADM and HGF support migration of MPNST cells, and NF1 MPNST cell conditioned medium supports endothelial cell tube formation. A, NF1-associated MPNST cell migration is stimulated by ADM and by MPNST serum-free conditioned medium. B, sporadic MPNST cell migration is stimulated by ADM and MPNST serum-free conditioned medium. ADM was used at 10 μmol/L. ADM 22-52 (Inhib) was used at 10 μmol/L. Student's two-tailed *t* test compared controls with each individual experimental group. C, NF1-associated MPNST conditioned medium stimulates HUVEC tube formation. D, sporadic MPNST conditioned medium does not stimulate HUVEC tube formation compared with controls. EGM2, HUVEC base medium including serum, fibroblast growth factor, and platelet-derived growth factor; DMEM, serum-free DMEM; 8814 CM, serum-free conditioned medium (NF1-associated MPNST cell line); 26T CM, serum-free conditioned medium (sporadic MPNST cell line); ADM, 10 μmol/L ADM; ADM inhib, 10 μmol/L ADM 22-52; HGF, 10 ng/mL HGF; HGF inhib, 0.5 μg/mL HGF inhibitor.

ovarian carcinomas (47). In 2005, Huddleston et al. discovered that creatine kinase B (CKB) is overexpressed in ovarian carcinoma cell lines compared with normal cell lines on microarray analysis. ELISA-based assays were also subsequently done on serum samples from patients with ovarian carcinoma, patients with benign pelvic masses, and normal controls. CKB gene expression was found to be upregulated in ovarian cancer cells *in vitro* and *in vivo* and that CKB enzyme activity is significantly elevated in sera from ovarian cancer patients, suggesting a potential role for CKB as a marker for early diagnosis (48). Although the use of microarray technology to elucidate a serum biomarker is not a novel concept, this is the first report in the literature of the use of a microarray-generated biomarker specific for NF1 and MPNST.

Other studies have documented increased serum levels of ADM in neuroendocrine tumors (49). However, there are no previous literature reports of ADM being studied in the serum of human sarcomas or NF1-associated tumors. We found that serum levels of ADM are elevated in patients with NF1 regardless of tumor status, similar to a previous report of fetal antigen 1 (18). In addition, because ADM levels were significantly higher in NF1 patients with MPNST than pNF, our data suggest that ADM may be a serum biomarker for malignant transformation. Larger studies should be done to confirm these findings as well as compare these

markers to other prognostic tools such as [^{18}F]2-fluoro-2-deoxy-D-glucose positron emission tomography scans.

ADM might also be relevant to tumor growth. However, the amounts of ADM required to stimulate tube formation and migration experiments are higher that those identified in patient serum. We have not evaluated the local concentration in the tissue to the concentrations used *in vitro*, yet we note that concentrations in dense cells *in vitro* are high enough to generate effects on migration and tube formation. In addition, the 26T MPNST cells are not stimulated to grow by NF1-specific 8814 MPNST conditioned medium, whereas 26T MPNST conditioned medium is able to stimulate migration in 8814 MPNST cells. This discrepancy could be due to NF1-specific MPNST cells being more sensitive to the effects of ADM, a hypothesis further reinforced in the tube formation experiments. A limitation of this study is the small MPNST sample size; a larger series of MPNST patients' sera should be screened to validate these findings. Another limitation to our data interpretation is the use of surplus sera as controls. The characteristics of these sera were not fully defined apart from age and gender. Although we excluded patients with other tumors, the control sera used for this analysis could have come from patients who may have had other disease processes, such as liver disease or urinary tract infections (50, 51), which may have abnormal levels of ADM and/or HGF. In addition, only four of eight MPNST cell lines were viable in serum-free medium. As such, results using conditioned medium may be biased toward a subset of MPNSTs that may represent a biologically meaningful subgroup. However, the microarray data showed an increase in expression across all neurofibromas and MPNSTs, which argues against the possibility of such bias.

HGF did not prove to be a serum biomarker in NF1 patients with tumors, although HGF was highly expressed in our transcriptome analysis. Although serum levels in NF1 patients did not suggest its use as a biomarker, HUVEC tube formation data support the idea that HGF contributes to growth of MPNST *in vivo* via stimulation of angiogenesis. HUVEC tube formation was inhibited by the addition of ADM and HGF antagonists in the presence of NF1-associated MPNST serum-free conditioned medium, confirming that MPNSTs secrete angiogenic factors, including HGF and ADM. We have shown that NF1-specific MPNST serum-free conditioned medium contains higher levels of ADM and HGF than sporadic MPNST conditioned medium. Unlike sporadic MPNST serum-free conditioned medium, NF1-associated MPNST serum-free conditioned medium stimulates tube formation in HUVECs. Additionally, ADM was shown to have specific MPNST cell-autonomous effects on cell migration. These findings identify the potential involvement of ADM in NF1-associated MPNST pathology and reinforce the potential involvement HGF (23, 24). Further confirmatory investigation is warranted, as treatment with anti-HGF antibodies could be a potential therapeutic option for NF1-associated MPNST, especially as the use of monoclonal antibodies to HGF for treatment of HGF/c-Met–dependent tumors is in clinical phase I trials (52).

In summary, we have shown the feasibility of integrating gene expression data with known or predicted secreted proteins to select potential NF1 biomarkers. We identify one candidate, ADM, as a biomarker for pNF and NF1-associated MPNST. Larger prospective studies will be required to confirm this finding and rigorously test further elevation of ADM serum levels in patients with MPNSTs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were identified.

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References

1. Rasmussen SA, Friedman JM. NF1 gene and neurofibromatosis 1. Am J Epidemiol 2000;151:33–40.
2. Legius E, Marchuk DA, Collins FS, Glover TW. Somatic deletion of the neurofibromatosis type 1 gene in a neurofibrosarcoma supports a tumour suppressor gene hypothesis. Nat Genet 1993;3:122–6.
3. Listernick R, Louis DN, Packer RJ, Gutmann DH. Optic pathway gliomas in children with neurofibromatosis 1: consensus statement from the NF1 Optic Pathway Glioma Task Force. Ann Neurol 1997; 41:143–9.
4. Stiller CA, Chessells JM, Fitchett M. Neurofibromatosis and childhood leukaemia/lymphoma: a population-based UKCCSG study. Br J Cancer 1994;70:969–72.
5. Colman SD, Williams CA, Wallace MR. Benign neurofibromas in type 1 neurofibromatosis (NF1) show somatic deletions of the NF1 gene. Nat Genet 1995;11:90–2.
6. Evans DG, Baser ME, McGaughran J, Sharif S, Howard E, Moran A. Malignant peripheral nerve sheath tumours in neurofibromatosis 1. J Med Genet 2002;39:311–4.
7. Zoller ME, Rembeck B, Oden A, Samuelsson M, Angervall L. Malignant and benign tumors in patients with neurofibromatosis type 1 in a defined Swedish population. Cancer 1997;79:2125–31.
8. Helman LJ, Meltzer P. Mechanisms of sarcoma development. Nat Rev Cancer 2003;3:685–94.
9. McCaughan JA, Holloway SM, Davidson R, et al. Further evidence of the increased risk for malignant peripheral nerve sheath tumour from a Scottish cohort of patients with neurofibromatosis type 1. J Med Genet 2007;44:463–6.
10. Miser JS, Triche TJ, Kinsella TJ, Pritchard DJ. Other soft tissue sarcomas of childhood. 4th ed. Philadelphia (PA): Lippincott Williams &Wilkins; 2002.
11. Ferner RE, Golding JF, Smith M, et al. [^{18}F]2-fluoro-2-deoxy-D-glucose positron emission tomography (FDG PET) as a diagnostic

tool for neurofibromatosis 1 (NF1) associated malignant peripheral nerve sheath tumours (MPNSTs): a long-term clinical study. Ann Oncol 2008;19:390–4.
12. International Germ Cell Cancer Collaborative Group. International germ cell consensus classification: a prognostic factor-based staging system for metastatic germ cell cancers. J Clin Oncol 1997;15:594–603.
13. Frankel S, Smith GD, Donovan J, Neal D. Screening for prostate cancer. Lancet 2003;361:1122–8.
14. Ishikawa O, Ishikawa H. Neurofibroma-derived fibroblast-stimulating factor in the serum of patients with neurofibromatosis (von Recklinghausen's disease). Arch Dermatol Res 1985;277:433–8.
15. Riccardi VM. Growth-promoting factors in neurofibroma crude extracts. Ann N Y Acad Sci 1986;486:206–26.
16. Riopelle RJ, Riccardi VM, Faulkner S, Martin MC. Serum neuronal growth factor levels in von Recklinghausen's neurofibromatosis. Ann Neurol 1984;16:54–9.
17. Mashour GA, Ratner N, Khan GA, Wang HL, Martuza RL, Kurtz A. The angiogenic factor midkine is aberrantly expressed in NF1-deficient Schwann cells and is a mitogen for neurofibroma-derived cells. Oncogene 2001;20:97–105.
18. Jensen CH, Schroder HD, Teisner B, Laursen I, Brandrup F, Rasmussen HB. Fetal antigen 1, a member of the epidermal growth factor superfamily, in neurofibromas and serum from patients with neurofibromatosis type 1. Br J Dermatol 1999;140:1054–9.
19. Miller SJ, Rangwala F, Williams J, et al. Large-scale molecular comparison of human schwann cells to malignant peripheral nerve sheath tumor cell lines and tissues. Cancer Res 2006;66:2584–91.
20. Moriyama T, Kataoka H, Kawano H, et al. Comparative analysis of expression of hepatocyte growth factor and its receptor, c-met, in gliomas, meningiomas and schwannomas in humans. Cancer Lett 1998;124:149–55.
21. Grant DS, Kleinman HK, Goldberg ID, et al. Scatter factor induces blood vessel formation *in vivo*. Proc Natl Acad Sci U S A 1993;90: 1937–41.
22. Su W, Gutmann DH, Perry A, Abounader R, Laterra J, Sherman LS. CD44-independent hepatocyte growth factor/c-Met autocrine loop promotes malignant peripheral nerve sheath tumor cell invasion *in vitro*. Glia 2004;45:297–306.
23. Rao UN, Sonmez-Alpan E, Michalopoulos GK. Hepatocyte growth factor and c-MET in benign and malignant peripheral nerve sheath tumors. Hum Pathol 1997;28:1066–70.
24. Fukuda T, Ichimura E, Shinozaki T, et al. Coexpression of HGF and c-Met/HGF receptor in human bone and soft tissue tumors. Pathol Int 1998;48:757–62.
25. Krasnoselsky A, Massay MJ, DeFrances MC, Michalopoulos G, Zarnegar R, Ratner N. Hepatocyte growth factor is a mitogen for Schwann cells and is present in neurofibromas. J Neurosci 1994; 14:7284–90.
26. Kitamura K, Kangawa K, Kawamoto M, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun 1993;192:553–60.
27. Zudaire E, Martinez A, Cuttitta F. Adrenomedullin and cancer. Regul Pept 2003;112:175–83.
28. Hague S, Zhang L, Oehler MK, et al. Expression of the hypoxically regulated angiogenic factor adrenomedullin correlates with uterine leiomyoma vascular density. Clin Cancer Res 2000;6:2808–14.
29. Iimuro S, Shindo T, Moriyama N, et al. Angiogenic effects of adrenomedullin in ischemia and tumor growth. Circ Res 2004;95:415–23.
30. Kaur B, Khwaja FW, Severson EA, Matheny SL, Brat DJ, Van Meir EG. Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis. Neuro-oncol 2005;7:134–53.
31. Ouafik L, Sauze S, Boudouresque F, et al. Neutralization of adrenomedullin inhibits the growth of human glioblastoma cell lines *in vitro* and suppresses tumor xenograft growth *in vivo*. Am J Pathol 2002; 160:1279–92.
32. Chen Y, Zhang Y, Yin Y, et al. SPD—a web-based secreted protein database. Nucleic Acids Res 2005;33:D169–73.
33. Miller SJ, Jessen WJ, Mehta T, et al. Integrative genomic analyses of neurofibromatosis tumors identify SOX9 as biomarker and survival gene. EMBO Mol Med 2009;1:236–48.
34. Irizarry RA, Hobbs B, Collin F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003;4:249–64.
35. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. Behav Brain Res 2001; 125:279–84.
36. Jiang WG, Martin TA, Parr C, Davies G, Matsumoto K, Nakamura T. Hepatocyte growth factor, its receptor, and their potential value in cancer therapies. Crit Rev Oncol Hematol 2005;53:35–69.
37. Zimmermann U, Fischer JA, Frei K, Fischer AH, Reinscheid RK, Muff R. Identification of adrenomedullin receptors in cultured rat astrocytes and in neuroblastoma × glioma hybrid cells (NG108-15). Brain Res 1996;724:238–45.
38. Martinez A, Vos M, Guedez L, et al. The effects of adrenomedullin overexpression in breast tumor cells. J Natl Cancer Inst 2002;94:1226–37.
39. Oehler MK, Norbury C, Hague S, Rees MC, Bicknell R. Adrenomedullin inhibits hypoxic cell death by upregulation of Bcl-2 in endometrial cancer cells: a possible promotion mechanism for tumour growth. Oncogene 2001;20:2937–45.
40. Ishikawa T, Chen J, Wang J, et al. Adrenomedullin antagonist suppresses *in vivo* growth of human pancreatic cancer cells in SCID mice by suppressing angiogenesis. Oncogene 2003;22:1238–42.
41. Hay DL, Howitt SG, Conner AC, Schindler M, Smith DM, Poyner DR. CL/RAMP2 and CL/RAMP3 produce pharmacologically distinct adrenomedullin receptors: a comparison of effects of adrenomedullin22-52, CGRP8-37 and BIBN4096BS. Br J Pharmacol 2003;140:477–86.
42. Kim W, Moon SO, Sung MJ, et al. Angiogenic role of adrenomedullin through activation of Akt, mitogen-activated protein kinase, and focal adhesion kinase in endothelial cells. FASEB J 2003;17:1937–9.
43. Morimoto A, Okamura K, Hamanaka R, et al. Hepatocyte growth factor modulates migration and proliferation of human microvascular endothelial cells in culture. Biochem Biophys Res Commun 1991; 179:1042–9.
44. Assimakopoulou M, Sotiropoulou-Bonikou G, Maraziotis T, Papadakis N, Varakis I. Microvessel density in brain tumors. Anticancer Res 1997; 17:4747–53.
45. Simpson A, Grimer R, Mangham C, Cullinane C, Lewis I, Burchill SA. MVD predicts disease-free and overall survival in tumours of the Ewing's sarcoma family (ESFT). Br J Cancer 2002;86:P202.
46. Dhanasekaran SM, Barrette TR, Ghosh D, et al. Delineation of prognostic biomarkers in prostate cancer. Nature 2001;412:822–6.
47. Mok SC, Chao J, Skates S, et al. Prostasin, a potential serum marker for ovarian cancer: identification through microarray technology. J Natl Cancer Inst 2001;93:1458–64.
48. Huddleston HG, Wong KK, Welch WR, Berkowitz RS, Mok SC. Clinical applications of microarray technology: creatine kinase B is an up-regulated gene in epithelial ovarian cancer and shows promise as a serum marker. Gynecol Oncol 2005;96:77–83.
49. Pavel ME, Hoppe S, Papadopoulos T, et al. Adrenomedullin is a novel marker of tumor progression in neuroendocrine carcinomas. Horm Metab Res 2006;38:112–8.
50. Dotsch J, Hanze J, Knufer V, et al. Increased urinary adrenomedullin excretion in children with urinary tract infection. Nephrol Dial Transpl 1998;13:1686–9.
51. Hsieh CS, Huang LT, Lee SY, et al. Evaluation of hepatocyte growth factor in patients with biliary atresia. J Pediatr Surg 2008;43:1333–7.
52. Burgess T, Coxon A, Meyer S, et al. Fully human monoclonal antibodies to hepatocyte growth factor with therapeutic potential against hepatocyte growth factor/c-Met-dependent human tumors. Cancer Res 2006;66:1721–9.