Genomic and Molecular Characterization of Malignant Peripheral Nerve Sheath Tumor Identifies the IGF1R Pathway as a Primary Target for Treatment

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Abstract

Purpose: Malignant peripheral nerve sheath tumor (MPNST) is a rare sarcoma that lacks effective therapeutic strategies. We gain insight into the most recurrent genetically altered pathways with the purpose of scanning possible therapeutic targets.

Experimental Design: We conducted a microarray-based comparative genomic hybridization profiling of two cohorts of primary MPNST tissue samples including 25 patients treated at The University of Texas MD Anderson Cancer Center and 26 patients from Tianjin Cancer Hospital. Immunohistochemistry (IHC) and cell biology detection and validation were carried out on human MPNST tissues and cell lines.

Results: Genomic characterization of 51 MPNST tissue samples identified several frequently amplified regions harboring 2,599 genes and regions of deletion including 4,901 genes. At the pathway level, we identified a significant enrichment of copy number–altering events in the insulin-like growth factor 1 receptor (IGF1R) pathway, including frequent amplifications of the IGF1R gene itself. To validate the IGF1R pathway as a potential target in MPNSTs, we first confirmed that high IGF1R protein correlated with worse tumor-free survival in an independent set of samples using IHC. Two MPNST cell lines (ST88-14 and STS26T) were used to determine the effect of attenuating IGF1R. Inhibition of IGF1R in ST88-14 cells using siRNAs or an IGF1R inhibitor, MK-0646, led to significant decreases in cell proliferation, invasion, and migration accompanied by attenuation of the PI3K/AKT and mitogen-activated protein kinase pathways.

Conclusion: These integrated genomic and molecular studies provide evidence that the IGF1R pathway is a potential therapeutic target for patients with MPNST.

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Introduction

Malignant peripheral nerve sheath tumors (MPNST), a subtype of soft-tissue sarcomas of neural crest origin (1), are highly malignant and account for approximately 5% to 10% of all soft-tissue sarcomas (2). Currently, the 5-year survival rates of MPNST patients are still only 30% to 50%, even with multidisciplinary treatments such as aggressive surgery, high-dose adjuvant chemotherapy, and radiotherapy (1). The dismal outcome not only points to the urgent need to establish better therapeutic strategies for patients harboring MPNSTs but also highlights the importance of exploring the genomic basis of the disease to identify recurrent oncogenic events for targeted therapy.

A number of large cancer genome characterization efforts have already proven the value of the genomic approach by identifying several new therapeutic targets and giving insights into general cancer biology (3). However, such large projects concentrate on common cancers that have a high incidence and prevalence. For rare types of cancers,
Translational Relevance

A major contribution of our study is the characterization of insulin-like growth factor 1 receptor (IGF1R) as a potential therapeutic target for MPNST patients by genomic, immunohistochemistry (IHC), and cell biology approaches. We present a comprehensive characterization of a large cohort of 51 primary tumors using comparative genomic hybridization technology resulting in a map of the MPNST genome. Furthermore, we apply pathway-level analyses that provide a unique view into the aberrant signaling networks in MPNST, which we then proceed to validate with IHC and cell biology approaches in MPNST cell lines. These integrated genomic and molecular studies provide evidence that IGF1R is a promising therapeutic target in MPNST patients. This is the first time that the genetic aberrations of important signaling pathways have been investigated with the purpose of scanning possible therapeutic targets in MPNST. Extensive investigations of these pathways might give added confidence to move translational research results to the clinics to benefit patients with MPNST.

Materials and Methods

Ethics statement

All of the tissue and information collection took place at Tianjin Medical University Cancer Hospital and MD Anderson Cancer Center with Institutional Review Board (IRB) approved protocols and the patients' consent.

Primary tumors

 Archived MPNST samples and matching patient records were acquired from The University of Texas MD Anderson Cancer Center (25 formalin-fixed paraffin-embedded, FFPE, tumor samples) and Tianjin Cancer Hospital of China (26 fresh-frozen tumor samples; Supplementary Table S1). All samples had at least 90% tumor content. In addition, we acquired 36 FFPE tumor samples for immunohistochemical validations (Supplementary Table S2). All samples were obtained with the approval of the IRBs of the 2 institutions. Patient records included age, sex, tumor location, tumor size (largest diameter of the tumor), American Joint Committee on Cancer (AJCC) stage of the tumor, time to recurrence, metastatic status, treatments administered, and follow-up outcomes. The presence or absence of NF1 syndrome was determined on the basis of established NIH criteria (10). MPNST patients received chemotherapy after the primary tumor excision using a regimen of mesna, doxorubicin, ifosfamide, and dacarbazine. When indicated, 30 to 60 Gy of radiotherapy was administered to the surgical region and/or metastatic lesions. The range of surgical operations included wide and subtotal (including subtotal wide, marginal, and intraleisional) resections.

Array CGH hybridization

Genome-wide copy number measurements were made for 51 primary tumor samples. Commercially available normal genomic DNAs were used as control (Clontech Laboratories, Inc.). All the surgical samples were collected before radiation treatment. Genomic DNA was isolated according to standard procedures. Labeled genomic DNA was hybridized using an Agilent Human Genome CGH Microarray kit (4 × 44 k; Agilent Technologies). These arrays represent more than 43,000 coding and noncoding human sequences yielding an average of 35-kbp oligonucleotide probe spatial resolution. At least 1 target sequence was analyzed for every well-characterized gene, and at least 2 target sequences were analyzed for every known cancer gene. The probes were designed based on the University of California Santa Cruz hg17 human genome (National Center for Biotechnology build 35, May 2004).
The processing of the aCGH data and the frequency analyses were carried out as described previously (11). Briefly, the ratios of intensity values from tumor and normal tissues were transformed to log₂-space. Log ratio data were then subjected to a circular binary segmentation algorithm to reduce the effect of noise. After that, the CGHcall algorithm was used to give each segment an aberration label: normal, deletion, or amplification. An aberration frequency for each probe was established by combining the labels from individual samples.

**IHC methods**

Fifty-six FFPE tissues from Tianjin Cancer Hospital were cut into 4-μm sections and mounted on charged glass slides (ProbeOn Plus; Fisher Scientific) for IHC analyses according to published methods (12–14). IGFIR antibody was used in 1:75 dilutions (Santa Cruz Biotechnology, Santa Cruz, CA). The same concentrations of nonimmune rabbit or goat serum were used as negative controls. The expression levels of IGF1R were estimated according to the criteria previously reported (12–14). Scoring was carried out according to the percentage of positive cells: <5% was classified as negative (−), 6% to 30% was classified as a weak positive (+), 31% to 60% as a moderate positive (++), and >60% as a strong positive (+++).

**Cell culture and compounds**

The MPNST cell lines ST88-14 and STS26T were maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. Cells were incubated at 37°C in a humidified atmosphere of 7.5% CO₂. Authentication of these 2 MPNST cell lines was conducted utilizing short tandem repeat (STR) DNA fingerprinting. The ST-8814 line is NF1+/−, whereas STS26T is NF1+/+. IGFIR monoclonal antibody MK-0646 (obtained from Merck) was dissolved in sterile water at a concentration of 20 mg/mL and stored at −20°C. Gefitinib was stored as a 20 mmol/L stock solution in dimethyl sulfoxide.

**siRNA and plasmid transfections**

For the siRNA studies, a smart pool of 3 double-stranded siRNAs against IGF-1R (IGF1R-NM-000875) was obtained from Dharmacon Tech and used according to the manufacturer's instructions and this siRNA smart pool has been proven specific and effective in previous reports (12–16). Because some reports have reported cross-talk between the IGF1R and epidermal growth factor receptor (EGFR) signal pathways (17–19), a previously proven specific and effective EGFR siRNA (sc-29301, Santa Cruz Biotechnology) was also used both individually and combined with siRNA for IGF-1R as described previously (12–16). Nonspecific siRNA (D-001206-01-05) was obtained from Dharmacon Tech was used as a control in all experiments (16). To generate IGF1R expression vectors, the IGF1R cDNA insert was digested by EcoR1 and then ligated to the pCDNA3.1(+) vector. Positive clones were verified by sequencing. The transfection of plasmid DNA were carried out as described previously (13, 14, 20, 21).

**Western blot analysis and cell proliferation, invasion, and migration assays**

Western blot analysis was carried out according to standard procedures as described previously (22). The antibodies for EGFR, AKT, PI3K, IRS-1, ERK, and their phosphorylated forms were purchased from Abcam, Santa Cruz, Cell Signaling, and Sigma and were used according to the manufacturers’ instructions. Cell proliferation was analyzed by MTT assay, and cell invasion and migration were analyzed by Transwell migration assays (EMD Biosciences) as previously described (22).

**Statistical analyses**

Clinical and pathologic characteristics of the 26 Chinese and 25 American MPNST patients were compared using χ² test (Supplementary Table S1). The associations between clinicopathologic and molecular characteristics and the survival were analyzed with Cox multiple regression models (Supplementary Table S2). The associations between CNAs and survival were computed with Mantel–Cox test of difference of Kaplan–Meier survival estimators (Supplementary Data S2). Associations between CNAs and other clinical variables (Supplementary Data S2) were computed using the Fisher’s exact test. This test was also used in comparing the differences (in gene level) between aberration profiles categorized by different clinical variables. Pathway enrichment analysis, using a standard hypergeometric test, was carried out on the genes that were either amplified or deleted in at least 25% of the samples. Enrichment P values were computed for all signaling pathways available in Biocarta (http://www.biocarta.com/). A P value of 0.05 was considered the threshold of statistical significance in all tests.

**Results**

**MPNSTs exhibit recurrent genetic aberrations that significantly alter multiple signaling pathways**

Integration of copy number profiles of the individual samples resulted in the discovery of several major regions of frequent deletions and amplifications in the 51 primary MPNST tissue samples (Fig. 1A). With approximately 65% of patients affected, we identified focal deletion of 9p21.3 (harboring tumor suppressors CDKN2A and CDKN2B) as the most recurrent genomic event in our data, consistent with a previous study (Ref. 9; Fig. 1B). Highly recurrent amplifications in 7p harboring EGFR, BRAF, ETF1, MET, AKAP9, 8q harboring MYC, ETT1, NCOA2, and 17q harboring BRIP1, CLTC, MIPI, PRKRA1A were also prominent (30%–40% frequency), as observed previously (Refs. 6–8; Fig. 1A). More novel chromosomal abnormalities included deletions of 1p, containing TP73 and MHP, 10q26 containing MGMT, 16p containing MMPI5, chromosome 19 with several cancer-related genes, including AKT2, BCL3, CEBPA, and ERCC2, and 22q containing GSTT1, MTL1, MYH9,
**NF2, PDGFβ, SMARCB1.** Previously unreported amplifications were identified in chromosomes 1q (with ASPM), 12q (with MDM2, KRAS, ETV6), and 15q (with IGF1R; Fig. 1A). In total, frequently amplified and deleted regions (aberrated in at least 25% of the samples) harbored 2,599 and 4,901 genes, respectively (Supplementary Data S1).

Subsequently, we investigated the translational relevance of these genes by correlating the loci with several clinical parameters such as AJCC, tumor size, local recurrence, and metastasis (Supplementary Data S2). For example, the amplification of MYC was significantly associated with tumor recurrence, and the deletion of AKT1 was associated with the presence of tumor metastases. Interestingly, we could not associate any individual aberration with patient survival, suggesting that multiple events might cooccur to affect survival. However,
correlating the overall frequency of CNAs with survival did not implicate increased genomic instability in inducing statistically significant survival effects. To investigate the genomic relevance of the clinical variables, we compared the effect of several parameters to the global aberration profiles. First, the samples were divided based on patient ethnicity. The most significant difference in the aberration profiles was the decreased overall aberration rate in the Chinese patients, yet the overall pattern of aberrations remained fairly similar. Second, comparisons of aberration profiles with respect to other clinical parameters such as tumors extracted from the trunk versus extremity resulted in a low number of loci that were aberrated with a significantly different rate.

To investigate the alterations at the signaling pathway level, we computed pathway enrichment scores in pathways described in Biocarta. This analysis resulted in 11 statistically significantly altered pathways (Fig. 1C). The most enriched pathway, TFF, is a mucosal healing pathway that contains parts of the ERK (the second most significant) and the EGFR pathways, both of which have been linked previously to MPNST (23). The third pathway, ARF, is the tumor-suppressor pathway in which CDKN2A (p16) plays a central role; this has been reported to be involved in the pathogenesis of MPNSTs (9, 24, 25). The 4th most significantly altered signaling pathway, the IGF1R signaling pathway, a major cell survival pathway, has not been previously reported in MPNST.

**Extensive IGF1R pathway alterations and increased IGF1R protein expression correlate with patient survival**

IGF1R amplification, which was amplified in 24% of our samples, is an attractive therapeutic target that has not been reported in MPNSTs (14, 26). Therefore, we investigated in greater depth the frequency and pattern of gene alterations in IGF1R signaling pathway (Fig 2A and B). In addition to IGF1R amplifications, at least 1 gene in the IGF1R pathway was altered in 82% of the cases making the pathway highly significant. Frequent deletions included MAPK1 (41%), H-RAS (35%), and PTEN (35%). Notably, the PTEN signaling pathway was also significantly altered in MPNSTs (Fig 2C). The most commonly amplified genes in the IGF1R pathway were BRAF (31%), GRB2 (31%), PIK3CG (37%), RPS6KB1 (31%), and EIF4EBP1 (33%).

Because IGF1R copy number status itself was not correlated with survival, we sought to determine whether there was a survival effect at the pathway level. We divided the samples into 2 groups based on the extent of IGF1R pathway alterations. One group was characterized by more than 10% of pathway genes altered, and the other group with less than 10% of altered genes (Fig. 2C). Interestingly, we found that the patients in the group with less alterations had a significantly better prognosis than the patients in the other group (P = 0.0379) (Fig. 2C). The alteration frequency in the IGF1R pathway correlated with the overall frequency of CNAs, but overall CNAs did not confer a significant survival effect. The 2 groups were also independent of the clinical parameter with prognostic significance identified in our analysis, the tumor size (Supplementary Table S2).

To further study the clinical importance of IGF1R, we analyzed the extent of IGF1R protein expression in an independent set of 56 FFPE MPNST tissue samples with an immunohistochemical assay (Supplementary Table S2). The protein expression of IGF1R exhibited various patterns, from negative and weak positives to moderate and strong positives, with a total positive rate of 82.1% (46/56; Fig. 2D, upper panel). Clinically, patients with an increased IGF1R protein expression had significantly worse tumor-free survival rates and a higher risk of tumor progression (Fig. 2D, lower panel).

**IGF1R activation contributes to MPNST cell proliferation, migration, and invasion by the activation of PI3K and AKT pathway signaling**

Several lines of evidence indicate that IGF1R may potentially be a very interesting clinical target in MPNST: the IGF1R gene is frequently amplified, the IGF1R protein expression correlates with survival, there are significant alterations in the signaling pathway that also correlate with survival, and there are successful IGF1R inhibitors already available to treat other cancers (13, 26–31). To determine whether IGF1R is a potential therapeutic target in MPNST, we evaluated the effect of its inhibition using 2 in vitro cell culture systems, the ST88-14 and STS26T MPNST cell lines. Western blotting indicated that IGF1R was readily detectable in the ST88-14 cell line, but the STS26T cells showed no detectable IGF1R expression. In ST88-14 cells, the decrease in IGF1R expression caused by IGF1R siRNA significantly reduced expression of pIGF1R and other AKT/PI3K signaling pathway activators (Fig. 3A, left panel). Accordingly, IGF1R siRNA effectively blocked tumor cell proliferation (Fig. 3A, right panel), invasion (Fig. 3B), and migration (Fig. 3C). We next evaluated the effect of anti-IGF1R (MK-0646) agents that are being used clinically. In ST88-14 cells, treatment with MK-0646 led to a decrease in the activated form of IGF1R and a decrease in cell proliferation relative to control (Fig. 4A and B). These results suggested that IGF1R is potential therapeutic target in MPNST.

The lack of IGF1R expression in STS26T cells provided us with an opportunity to evaluate whether IGF1R expression exerted a stimulating effect on MPNST cell proliferation. We transfected the cells with an IGF1R expression vector and found a marked increase in the levels of phosphorylated IGF1R (pIGF1R), pAKT, phosphorylated IRS-1 (pIRS-1), and pERK in these cells. The cell proliferation assay showed an increased rate of cell growth after the addition of the IGF1R expression vector (Fig. 5A and B). Similarly, the effect of augmented IGF1R expression levels was also observed in transfected ST88-14 cells, which had detectable levels of endogenous IGF1R expression (Fig. 5C). Combining all these data, we suggest IGF1R is targetable because its activation contributes to MPNST cell proliferation, migration, and invasion by the activation of PI3K and AKT pathway signaling.
Targeting IGF1R and EGFR in combination does not result in additive anti-MPNST effects compared with using each agent alone.

Because cross-talk between the IGF1R and EGFR signaling pathways has been detected in other types of cancers (12, 17–19, 32), we wanted to evaluate the possibility of synergistic or antagonistic effects resulting from simultaneously blocking both IGF1R and EGFR in MPNSTs. Because ST88-14 cells expressed both IGF1R and EGFR, we investigated the effect of inhibiting these 2 signaling molecules individually and in combination. Similar to the effect of IGF1R blocking, the decreased EGFR expression caused...
by EGFR siRNA had an inhibitory effect on AKT/PI3K signaling activators and on cell proliferation (Fig. 4C). Notably, attenuation of IGF1R and EGFR by combined siRNAs in ST88-14 cells significantly decreased cell proliferation without noticeable additive or combinational synergistic effects (Fig. 4C). By using EGFR and IGF1R inhibitors, we noticed that treatment with gefitinib in ST88-14 cells led to a decrease in the activated forms of EGFR and a decrease in cell proliferation relative to control without the activation of IGF1R signal pathways (Fig. 4A and B). Most importantly, combined treatment with both inhibitors did not result in a stronger inhibition of cell proliferation even though the combined treatment led to a larger decrease in pAKT and pERK at the molecular level (Fig. 4B).

### Discussion

MPNST poses significant clinical challenges because it is a highly malignant tumor characterized by a high rate of local recurrence and a strong tendency to metastasize (33). The dismal prognosis highlights the importance of identifying new clinicopathologic and molecular factors that affect MPNST outcome and the urgent need to establish better therapeutic strategies for patients with MPNST. In this study, we conducted genomic and molecular studies of MPNST samples and found evidence that IGF1R protein overexpression is an important molecular marker for tumor-free survival in MPNST patients and that IGF1R is a promising therapeutic target in this disease.
Figure 4. Attenuated IGF1R and/or EGFR significantly inhibited cell proliferation in MPNST ST88-14 cells by blocking the PI3K/AKT and MAPK pathways. A, gefitinib and MK-0646 significantly decreased the activation of EGFR and IGF1R. Left, IC50 of MK-0646 and gefitinib in MPNST ST88-14 cells. Right, the inactivation of EGFR and IGF1R. B, gefitinib and/or MK-0646 blocked activation of the PI3K/AKT and MAPK pathways and the MPNST ST88-14 tumor cell proliferation. Left, the inactivation of the PI3K/AKT and MAPK pathways. Right, the inhabitation of the MPNST ST88-14 tumor cell proliferation. C, the IGF1R and/or EGFR siRNAs inhibited the activation of PI3K/AKT and MAPK pathways and tumor cell proliferation in ST88-14 MPNST cells. Left, the inhabitation of the activation of PI3K/AKT and MAPK pathways. Right, the inhabitation of ST88-14 tumor cell proliferation.
A major contribution of this study is the extensive characterization of IGF1R as a potential therapeutic target for MPNST patients by genomic, IHC, and cellular biologic approaches. Several lines of evidence implicate IGF1R as a potential therapeutic target in MPNST: the IGF1R gene is frequently amplified; the IGF1R protein expression correlates with survival; and there are significant alterations in the signaling pathway that also correlate with survival. IGF1R inhibitors have already been successfully used to treat some types of cancers (13, 26–31). IGF1R is a multifunctional tyrosine kinase receptor involved in several biologic processes, including cell proliferation, differentiation, DNA repair, and cell survival (14, 31, 34, 35). Aberrant activation of the IGF1/IGF1R axis has been associated with a worse prognosis in many tumors, including breast, gastric, and prostate cancers (36, 37). Furthermore, in pancreatic cancer and anaplastic thyroid carcinomas, IGF1R inhibitors were shown to also reduce vascularization and VEGF expression (38, 39). Therefore, IGF1R is a logical potential molecular target in several types of cancers including breast, cervical, non-small cell lung, and prostate cancers (23, 32, 40–48). However, IGF1R-targeted therapies for sarcomas lag behind those for other cancers at present. Inhibition of IGF1R activity by its tyrosine kinase inhibitor NVP-AEW541 or its siRNA led to cytotoxicity and apoptosis in GIST cell lines by blocking the AKT and mitogen-activated protein kinase (MAPK) pathway signaling. Furthermore, the combination of NVP-AEW541 and imatinib in GIST cell lines induced a strong cytotoxic response (13, 14). In MPNST, however, information about IGF1R expression, its prognostic significance, and the cytotoxic potential of IGF1R inhibition is still lacking. In this study, the aCGH profile characterized the significant genetic amplifications of IGF1R signaling pathway genes including IGF1R itself. The deregulation of expression of IGF1R is an independent prognostic factor for this type of sarcoma. In the cell line studies, IGF1R siRNA and monoclonal antibody MK-0646 inhibited MPNST proliferation, invasion, and migration by blocking the AKT and PI3K pathways.

The introduction of anti-IGF1R antibodies in clinical trials and the dramatic single-agent anti-IGF1R activity observed in sarcoma patients provided the initial excitement in the sarcoma community (49, 50). However, the benefit of this therapeutic approach does not extend to all patients, with phase II studies showing less promising responses than initially anticipated (50). A major mechanism of resistance to highly specific inhibitors of IGF-1R, either antibodies or tyrosine kinase inhibitors may involve enhanced insulin receptor (IR)-A homodimer formation and IGF-2 production (50). Furthermore, the sensitivity to IGF1R-targeted therapy might be sarcoma-type dependent because our preclinical study shows IGF1R-targeted therapy might be effective in treating MPNST patients. One possible explanation is different compensatory responses in different sarcoma types in response to IGF1R inhibition. For example, different from the results in other tumors (17–19), in our study the inhibition of IGF1R did not result in the activation of EGFR pathways in MPNST and the combined inhibition of IGF1R and EGFR did not show additive antitumor effects at the cellular level, suggesting lack of cross-talk between IGF1R and EGFR pathways in MPNSTs. These indicate that targeting IGF1R in MPNST might be more efficient than in other cancer types. Thus, despite the disappointing phase II data in some sarcoma types, this novel class of drugs may constitute an active treatment in a proportion of sarcoma patients, especially MPNSTs.

Our aCGH profile with a large MPNST cohort revealed several important genetic aberrations with clinical relevance. We found that many genetic aberration events had significant correlation with clinical parameters of MPNST patients, including AJCC staging, tumor size, local recurrence, and metastasis. Extensive investigations of these genetic events and these correlations would shed light on MPNST pathogenesis. Furthermore, the pathway analyses revealed several signaling pathway genes harboring frequent genetic aberrations such as the TFF, ERK, ARF, and other signaling pathways. This is the first time that the genetic aberrations of important signaling pathways have been investigated with the purpose of scanning possible therapeutic targets in MPNST. Extensive investigations of these pathways might give added confidence to move translational research results to the clinic to benefit patients with MPNST.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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