MMSET Is Highly Expressed and Associated with Aggressiveness in Neuroblastoma

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Abstract

MMSET (WHSC1/NSD2) is a SET domain–containing histone lysine methyltransferase the expression of which is deregulated in a subgroup of multiple myelomas with the t(4;14)(p16;q32) translocation associated with poor prognosis. Recent studies have shown that MMSET mRNA levels are increased in other tumor types as well. We have carried out immunohistochemical staining of tissue microarrays and found that MMSET protein is frequently and highly expressed in neuroblastoma (MMSET positive in 75% of neuroblastomas, n = 164). The expression level of MMSET in neuroblastomas was significantly associated with poor survival, negative prognostic factors, and metastatic disease. Moreover, a subset of neuroblastomas for which pre- and post-chemotherapy biopsies were available displayed a strong decrease in MMSET protein levels after chemotherapy. In agreement with neuroblastomas becoming more differentiated after treatment, we show that retinoic acid–induced differentiation of human neuroblastoma cells in vitro also leads to a strong decrease in MMSET levels. Furthermore, we show that the high levels of MMSET in normal neural progenitor cells are strongly downregulated during differentiation. Importantly, we show that MMSET is required for proliferation of neuroblastoma cells and brain-derived neural stem cells. Taken together, our results suggest that MMSET is implicated in neuroblastomagenesis possibly by supporting proliferation of progenitor cells and negatively regulating their differentiation. In this respect, MMSET might be a strong candidate therapeutic target in a subset of neuroblastomas with unfavorable prognosis. Cancer Res; 71(12); 4226–35. ©2011 AACR.

Introduction

Neuroblastic tumors are embryonal neural sympathetic system tumors, which arise in the adrenal gland or the sympathetic chain (1). These tumors derive from cells that migrate from the neural crest and give rise to the sympathetic nervous system. They display a broad range of differentiation spanning from more mature and benign (ganglioneuromas) to more immature and potentially malignant (ganglioneuroblastomas) to undifferentiated and always malignant (neuroblastomas) forms. On the basis of the relative proportion of cytodifferentiated ganglion cells, neuroblasts, and Schwann cells, the neuroblastic tumors have been classified as stroma-rich tumors, including ganglioneuromas and intermixed ganglioneuroblastomas, and stroma-poor tumors also known as neuroblastomas (2, 3). The majority of neuroblastomas and ganglioneuroblastomas occur in the first 5 years of life, whereas most ganglioneuromas affect patients that are older than 10 years (1). Rare neuroblastomas and ganglioneuroblastomas occurring in adolescents and adults are characterized by longer clinical course, but the final outcome of these tumors is poor regardless of age (4). Neuroblastoma represents the most common extracranial solid malignancy of childhood and is usually sporadic, but familial cases associated with germ line gene mutations, particularly in the ALK gene, have been identified (5, 6). In addition, many different genetic aberrations have been identified in neuroblastoma including the most frequent 17q gain, 1p36 loss, and N-MYC amplification (7). The prognosis of high-risk neuroblastoma remains poor despite intensive chemotherapy. It is therefore of great importance to obtain a more detailed molecular understanding of the disease and to suggest potentially better prognostic markers.
The MMSET/WHSC1/NSD2 gene located at chromosome 4p16.3 encodes a histone lysine methyltransferase (8, 9). It undergoes alternative splicing resulting in different protein isoforms including MMSET type I, MMSET type II, and RE-IIBP (Supplementary Fig. S1A; refs. 10, 11). Northern blotting studies have revealed that MMSET mRNA is highly expressed in embryonic tissues, especially those with high degrees of proliferation, whereas in adult tissues, MMSET mRNA is primarily expressed in thymus and testis (10, 12, 13). MMSET is involved in the reciprocal t(4;14)(p16;q32) translocation in many human tumors (24). In this study, we show that MMSET is highly expressed in aggressive neuroblastic tumors collected in Italy (14–17). Recently, it was shown that MMSET is essential for growth of myeloma cell lines, suggesting a role of MMSET in proliferation of the malignant plasma cells (18–20). Upregulation of MMSET protein was found in glioblastoma versus normal brain cortex by proteome profiling (21). Other studies have also described high levels of MMSET mRNA in hematopoietic and neural cancers, with characteristics of radial glial cells and then further into neurons for 5 days as described (26).

To investigate MMSET protein expression in human tumors, we have generated a monoclonal antibody (mAb) that specifically reacts with MMSET in formalin-fixed, paraffin-embedded (FFPE) tissues. Through tissue microarray (TMA)-based screening for MMSET expression in more than 3,000 different tumor samples, we have found that MMSET is highly expressed in many human tumors (24). In this study, we show that MMSET is highly expressed in aggressive neuroblasticomas, is required for proliferation of neuroblastoma cells in tissue culture, and is involved in differentiation of neural progenitor cells (NPC).

Materials and Methods

Production of mAbs against MMSET

The MMSET fragment antigens Ag1 and Ag2 (corresponding to amino acids 1–119 and 939–985 of human MMSET type II, respectively) and the corresponding MMSET mAbs, 9A6 and 8C3, are schematically represented in Supplementary Fig. S1A. The development and characterization of 9A6 mAb has been described (24). The 9A6 mAb was effectively used for immunohistochemistry and both 8C3 and 9A6 mAbs were used for immunoblotting. The 8C3 mAb, but not the 9A6 mAb, recognizes mouse Mmset type II as well as human MMSET type II.

Tissue culture and differentiation protocols

All cells were cultured in a humidified incubator at 37°C with 5% CO2. U2OS cells were grown in Dulbecco’s modified Eagle medium (DMEM; Gibco) with 10% FBS (Hyclone) and 100 μg/ml penicillin and streptomycin (Gibco). Myeloma cell lines were grown in RPMI 1640 medium (Gibco) with 10% FBS (Hyclone) and 100 μg/ml penicillin and streptomycin (Gibco). SH-SY5Y cells were grown on collagen-coated plates (BD Biosciences) in minimum essential medium (Gibco) with 10% FBS (Hyclone) and 100 μg/ml penicillin and streptomycin (Gibco). For SH-SY5Y differentiation, 10⁶ cells were seeded on a 10-cm plate; 10 μmol/L retinoic acid (RA; Sigma-Aldrich) was added for 4 days and subsequently, 50 ng/ml brain-derived neurotrophic factor (BDNF; Invitrogen) for an additional 4 days in the absence of serum as described (25). SK-N-BE2 cells were grown in DMEM/F12 medium (Gibco) with 15% FBS (Hyclone) and 100 μg/ml penicillin and streptomycin (Gibco).

Mouse embryonic stem (ES) cells were differentiated into NPC with characteristics of radial glial cells and then further into neurons for 5 days as described (26).

Brain-derived neural stem cells (bNSC) were isolated from E12.5 mouse embryos and propagated as described in Supplementary Information. The bNSCs were differentiated by removing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) from the medium and adding 1% FBS (Hyclone). The pCAG MMSET overexpression construct was electroporated into bNSCs using the Nucleofector (Amaxa) and the Mouse Neural Stem Cell Kit (Amaza).

Colony assays

For colony assays, SH-SY5Y, SK-N-BE2, or bNSC cells were infected with MMSET pLKO knockdown constructs, incubated for 2 days, and selected in puromycin for 3 days. Subsequently, 2 × 10⁵ SH-SY5Y cells per well, 2 × 10⁵ SK-N-BE2 cells per well, and 2 × 10⁵ bNSC cells per well were seeded in a 6-well dish in triplicates. Colonies were stained with crystal violet.

TMA and staining with MMSET antibody

The initial neuroblastoma cohort (cohort 1, n = 44) was analyzed as a part of a large screening for MMSET expression carried out on TMA containing 3,818 FFPE representative archival samples (diameter, 0.6 mm) from many different human tumor types and 904 corresponding controls from normal tissues (24, 27). These samples (provided by R. Simon and G. Sauter) were derived from tissues previously collected for routine diagnostic procedures carried out at the Department of Pathology, University Medical Center Hamburg-Eppendorf (Germany) in accordance with the principles of the Ethik-Kommission der Ärztekammer, Hamburg.” Verification of results from cohort 1 was carried out on other TMAs containing a new cohort of FFPE, 1-mm large samples (cohort 2, n = 189) from neuroblastoma tumors collected in Italy (n = 67, diagnosed between 1992 and 2004, provided by R. Rota and M.A. De Ioris) and Denmark (n = 94, as previously described in ref. 1 and 28 additional tumors diagnosed between 2002 and 2010, provided by B.L. Petersen and E. Santoni-Rugiu). The 189 neuroblastoma tumors included 143 biopsies from primary tumors, 26 biopsies from metastatic tumors, and 20 biopsies taken after chemotherapy. The study was approved by the Institutional Review Board and Ethics Committee. Collection and TMA-based screenings of tumor samples were in accordance with the ethical principles for medical research issued by the World Medical Association’s Declaration of Helsinki. The screenings were not used to modify the original diagnosis or patients’ treatment.

The 143 primary neuroblastic tumors in cohort 2 comprised ganglioneuromas (n = 13), ganglioneuroblastomas (n = 10),
and neuroblastomas (n = 120). Corresponding clinicopathologic data and long-term follow-up were available for the patients in cohort 2 (Supplementary Fig. S2).

MMSET immunostaining was carried out on deparaffinized, 4-μm thick TMA sections by initially retrieving antigens in Tris-EGTA (TEG; pH = 9) buffer in a microwave oven (600 Watt/18 min), cooling down the slides for 20 minutes in dH2O, and thereafter blocking endogenous peroxidase activity according to the protocol of EnVision mouse kit (Dako). The TMA samples were incubated with purified 9A6 mAb (0.5 μg/μL, diluted 1:100) in 1% BSA (bovine serum albumin)/TBS buffer at 4°C overnight. As secondary antibody, EnVision mouse kit (Dako) was used. Immune reaction was revealed by 3,3′-diaminobenzidine (DAB; Dako) and CuSO4 to enhance the signal. As reference staining, germinal centers from hyperplastic lymph nodes were used (corresponding to MMSET score 2). Omission and substitution of 9A6 mAb with unspecific Ig was utilized as negative control. The MMSET immunostainings were analyzed by 2 pathologists (E. Santoni-Rugiu and B.L. Petersen) blind to clinicopathologic data and were scored as follows:

0 (negative): no nuclear staining
1 (weak): <10% positive cells (and weak nuclear staining intensity)
2 (moderate): between 10% and 50% positive cells (and moderate nuclear staining intensity)
3 (strong): >50% positive cells (and strong nuclear staining intensity)

The terms in parentheses indicate that, in the vast majority of the samples, a correlation was observed between amount of positive cells and intensity of the nuclear staining. A minimum of 100 relevant cells were counted. Uninformative TMA samples (fallen off during processing; poor quality due to artifact; not representative; fewer than 100 tumor cells in the sample) were excluded from the scores.

Statistics
All statistical analyses were carried out using Fisher’s exact test, Wilcoxon signed rank test, Kaplan–Meier log-rank test, and Cox regression analyses. P < 0.05 was considered significant.

Additional methods are available online, including plasmid constructs, primer sequences for quantitative real-time PCR (qRT-PCR), antibodies used for immunoblotting, and additional tissue culture information.

Results
MMSET is highly expressed in a subset of neuroblastomas
To determine the expression levels of MMSET in primary human tumors, 2 mAbs to MMSET, 9A6 and 8C3, recognizing different epitopes of the protein were generated (Supplementary Fig. S1A). The 9A6 mAB recognizes MMSET type I and II and the characterization of this antibody has been described (24). The 8C3 mAb recognizes MMSET type II and RE-IIBP and the specificity of 8C3 was shown by immunoblotting (Supplementary Fig. S1B). No expression of endogenous RE-IIBP was observed in U2OS cells, whereas a band presumably corresponding to RE-IIBP (but not confirmed by knockdown) was detected in several myeloma cell lines (Supplementary Fig. S1C).

The 9A6 mAb specifically detects MMSET in FFPE tissues and was used to determine MMSET protein expression levels in TMAs containing 3,818 biopsies from different types of human tumors and 904 samples from corresponding normal tissues (24). Among the different tumors, MMSET was highly expressed in adrenal neuroblastomas (cohort 1; n = 44; 68% positive) in contrast to an undetectable expression in normal adrenal gland (n = 12; P = 2.6 × 10−4). To validate these data from cohort 1, we tested a new cohort of samples representing the whole differentiation spectrum of neuroblastic tumors (cohort 2, n = 189). Given the relative rarity of these tumors, cohort 2 was represented by material collected from Danish and Italian patients, for whom corresponding clinicopathologic data (Supplementary Fig. S2) and long-term follow-up after diagnosis were available. This allowed clinical correlations with MMSET protein expression.

Cohort 2 included 143 primary biopsies from neuroblastic tumors of which 13 were ganglioneuromas, 10 ganglioneuroblastomas, and 120 neuroblastomas. In Fig. 1A, representative examples of immunostainings are shown with the MMSET score indicated. In Fig. 1B, the distribution among negative, weak, moderate, and strong MMSET expression for the ganglioneuromas, ganglioneuroblastomas, and neuroblastomas is shown. In the benign ganglioneuromas, no MMSET expression was detected (0 of 13, 0%), whereas 3 of 10 (30%) ganglioneuroblastoma samples had a detectable level of MMSET. In contrast, MMSET expression was observed in 93 of 120 (78%) neuroblastoma samples, the majority of which showed a MMSET score of 2 and 3. The difference in MMSET expression in primary neuroblastomas of cohort 2 with their corresponding clinicopathologic data (Supplementary Fig. S2) and long-term follow-up after diagnosis were available. This allowed clinical correlations with MMSET protein expression.

The MMSET expression pattern of primary neuroblastomas in cohort 2 was comparable with that observed in cohort 1, although with slight differences in the distribution into weak, moderate, and strong staining (Fig. 2A). However, overall, the 2 cohorts contained almost the same amount of MMSET-positive samples (68% vs. 78%). By combining the 2 cohorts into 1 pool, we obtained the most precise assessment of MMSET expression in neuroblastomas: 123 of 164 (75%) tumors were positive for MMSET expression and of these, 45 of 164 (27%) expressed MMSET at very high levels (score 3). We next correlated MMSET expression in primary neuroblastomas of cohort 2 with their corresponding clinicopathologic data. The patients with neuroblastomas displaying the highest MMSET expression levels (score 3) had significantly poorer survival than those with neuroblastomas showing lower or no expression (Fig. 2B; log-rank test,
Because no significant difference was observed in survival between patients with neuroblastoma with MMSET score 0, 1, and 2, these neuroblastomas were pooled in the following correlations to clinicopathologic data. The Shimada system is a standardized age-linked classification system for neuroblastomas dependent on the differentiation grade of the neuroblasts, their cellular turnover index, and the presence or absence of Schwannian stromal development. The tumors are assigned either a favorable or an unfavorable Shimada histology, based on these parameters (2, 3). A statistically significant difference in MMSET expression in neuroblastomas that were assigned a favorable versus an unfavorable Shimada score was observed, in that neuroblastomas with unfavorable Shimada score displayed higher MMSET expression (Table 1; Fisher’s exact test, \( P < 0.0001 \)). N-MYC amplification is present in approximately 30% of neuroblastoma cases, and this well-recognized genetic change is associated with advanced tumor stage and poor prognosis (28). Higher frequency and intensity of MMSET expression was observed in neuroblastomas with unfavorable Shimada score (Table 1; Fisher’s exact test, \( P = 0.008 \)). Furthermore, MMSET expression was lower in neuroblastomas from infants (<1 year of age) in comparison to neuroblastomas from older patients (>1 year of age; Table 1; Fisher’s exact test, \( P = 0.0051 \)). These data correlated with the fact that infants with neuroblastoma have a better prognosis than older patients (3). Interestingly, we also observed a significant inverse correlation between the degree of differentiation of the neuroblastomas and MMSET expression (Table 1; Fisher’s exact test, \( P = 0.0033 \)). This finding further supports a potential role of MMSET in differentiation. In conclusion, MMSET expression appears to correlate with well-known negative prognostic factors for neuroblastoma. Other clinical parameters, such as sex and tumor stage, were also correlated with MMSET expression. We observed numerical differences favoring a positive correlation of high MMSET expression with male patients and high stage, respectively, but they did not reach significance (Table 1; Fisher’s exact test, \( P = 0.084 \) and \( P = 0.056 \)). We also investigated whether MMSET expression was an independent prognostic factor in neuroblastoma, but that was not the case for our relatively small cohort in contrast to known independent prognostic factors such as age, stage, and N-MYC amplification (Supplementary Fig. S3).

In addition to biopsies from primary tumors, the TMAs from cohort 2 also included biopsies from metastases of neuroblastomas to solid organs (\( n = 12 \)) and to bone marrow (\( n = 14 \)). Both types of metastases, and particularly bone marrow metastases (Fisher’s exact test, \( P < 0.0001 \)), showed more frequent and stronger MMSET expression than primary tumors (Fig. 2C). The corresponding patient-matched biopsies from primary tumors were not available in most cases (either because neuroblastoma diagnosis was made on metastatic lesions or simply because it had not been possible to collect samples from primary tumors for the TMA). Nevertheless, the significant difference between MMSET expression in primary tumors and metastatic...
neuroblastomas suggests that MMSET could play a role in the late-stage progression of neuroblastoma to metastatic disease. MMSET expression is decreased following chemotherapy

For 20 neuroblastic tumors in cohort 2, biopsies taken after chemotherapy were available in addition to the primary biopsies taken at the time of diagnosis. These postchemotherapy biopsies contained representative vital tumor tissue, which was mostly characterized by a more differentiated phenotype than the one detectable in prechemotherapy biopsies (i.e., from prechemotherapy undifferentiated neuroblastoma to postchemotherapy poorly differentiated or differentiating neuroblastoma and from prechemotherapy neuroblastoma to postchemotherapy ganglioneuroblastoma), consistent with the often described maturation of neuroblastic tumors after chemotherapy (29). Interestingly, we observed a decrease in MMSET expression after treatment in 11 of 13 cases (85%) and in 6 of these cases, a cytologic differentiation was observed. In the remaining 2 cases and in the 7 MMSET-negative prechemotherapy cases, the MMSET expression levels remained unchanged after treatment. Representative examples of MMSET staining before and after chemotherapy are shown in Fig. 3A. The overall distribution of MMSET expression in the 20 neuroblastic tumors before and after chemotherapy is shown in Fig. 3B (Wilcoxon signed rank test, matched samples, \( P = 3.5 \times 10^{-3} \)). The decrease in MMSET expression after treatment paralleled in this way the induction of more differentiated tumor histology, an important observation with respect to the potential involvement of MMSET in differentiation of neuroblastic cells. These findings led us to consider the possibility that MMSET may negatively regulate differentiation of neuroblastic cells. To address this question, we investigated whether MMSET has a role in the regulation of differentiation of NPCs.

MMSET is involved in differentiation of NPCs

First, we investigated whether MMSET expression changes during various stages of neural differentiation. For this purpose, we used the human neuroblastoma SH-SY5Y cell line induced to differentiate by the addition of RA and BDNF. We monitored differentiation at days 0, 4, and 8 by assessing increasing neurite outgrowth (Supplementary Fig. S4), and by measuring the expression of the neuronal markers \( \beta\)-III-tubulin (30) and GAP43 (ref. 31; Fig. 4A, left). Concomitantly, we tested MMSET protein expression levels by immunoblotting and found that MMSET type II was drastically downregulated during differentiation, whereas the level of MMSET type I remained essentially unchanged (Fig. 4A, left). RE-IIBP protein was not detectable in SH-SY5Y cells (not shown). The downregulation of MMSET protein expression was not matched by a parallel decrease of MMSET type II mRNA levels (Fig. 4A, right). This indicates that the decrease of MMSET type II protein during differentiation of SH-SY5Y cells was due to a mechanism involving posttranscriptional regulation. MMSET type I and RE-IIBP mRNA levels did not significantly change either (Supplementary Fig. S5A). To investigate the type of posttranscriptional regulation, SH-SY5Y cells were differentiated with

![Figure 2. MMSET expression correlates with poor survival and metastasis of neuroblastoma. A, MMSET expression pattern in the primary neuroblastomas in cohort 1, cohort 2, and in a pool (cohort 1 + 2). B, Kaplan–Meier plot shows 5-year overall survival for patients with strong (score 3) versus no or lower MMSET expression (score 0, 1, and 2). C, MMSET expression in primary tumors versus solid and marrow metastases. These samples were not matched, as biopsies from primary tumors were missing from many of the patients with biopsies from metastasis.](image-url)
RA for 4 days and treated with the proteasome inhibitor MG-132. As the downregulation of MMSET type II protein was blocked by MG-132, MMSET type II protein is most likely targeted for proteolysis during differentiation of SH-SY5Y cells (Supplementary Fig. S6).

To understand whether MMSET levels are also decreased on differentiation of nontumorigenic cells, we determined the expression level of mouse Mmset type II, along with several differentiation markers, during in vitro differentiation of mouse ES cells. As shown in Fig. 4B, the expression level of mouse Mmset type II increased when mouse ES cells were differentiated into NPCs (day 0) and then decreased again when these cells were further differentiated into neurons (days 3–5). In accordance with a previous study (8), Mmset type II was also detectable in ES cells (Supplementary Fig. S5B). The increase in expression levels of mouse Mmset type II correlated with an increase in mRNA levels, when ES cells were differentiation into NPC (Fig. 4B, right). However, the drastic decrease in MMSET protein levels on differentiation of NPC into neurons was not paralleled by a similar reduction of Mmset type II mRNA (Fig. 4B, right). No detectable changes in Mmset type I mRNA levels were observed either (Supplementary Fig. S5C). Taken together, these results show that MMSET type II levels are dramatically reduced in differentiated neurons as compared with NPC, and that this decrease is due to posttranscriptional regulation of MMSET.

Given the high expression of MMSET in aggressive neuroblastomas, and the downregulation of MMSET during differentiation of a neuroblastoma cell line and NPC in vitro, we tested whether changing MMSET protein levels could affect proliferation and/or differentiation. Knockdown of MMSET type II in either SH-SY5Y or bNSCs led to a drastic inhibition in cell proliferation, as assessed by colony assays (Fig. 5A and B). The same inhibitory effect was also observed by knocking down MMSET type II in another neuroblastoma cell line, SK-N-BE2 that carries amplified N-MYC (Supplementary Fig. S7). In the converse experiment, ectopically expressed MMSET type II in bNSCs did not lead to an increase in proliferation; however, the differentiation of

| Table 1. Correlation of MMSET expression with clinicopathologic data in primary neuroblastomas of cohort 2 |
|---------------------------------|-----------|-----------|-------------|
| MMSET score | MMSET score | P (Fisher’s exact test) |
| 0 + 1 + 2 | 3 |
| Gender | | |
| Female | 43 | 8 | P = 0.084 |
| Male | 48 | 21 | |
| Age, y | | |
| <1 | 44 | 6 | P = 0.0051 |
| >1 | 47 | 23 | |
| Stage | | |
| Low (I, II, IV) | 43 | 8 | P = 0.056 |
| High (III, IV) | 46 | 21 | |
| Differentiation | | |
| Undifferentiated | 18 | 12 | P = 0.0033 |
| Poorly differentiated | 50 | 12 | |
| Differentiatiing | 23 | 5 | |
| Shimada | | |
| Favorable | 45 | 2 | P < 0.0001 |
| Unfavorable | 28 | 17 | |
| N-MYC | | |
| Single copy | 73 | 15 | P = 0.008 |
| Amplified | 14 | 13 | |

Figure 3. The expression of MMSET decreases in neuroblastic tumors treated with chemotherapy. For 20 neuroblastic tumors, biopsies taken after chemotherapy were included in the TMA in addition to the primary biopsies taken at the time of diagnosis. A, 2 representative examples of immunostainings of tumors before and after chemotherapy; ×40 magnification. B, distribution of MMSET expression for 20 matched pre- and postchemotherapy samples.
the cells were slightly delayed compared with control cells as measured by a higher expression of the stem cell–specific filament protein, Nestin (Fig. 5C, left; Supplementary Fig. S8). Interestingly, we were not able to maintain the overexpression of MMSET type II in the bNSCs, although high levels of the MMSET mRNA were present (Fig. 5C, right). Similarly to ectopically expressed MMSET, the endogenous mouse Mmset mRNA level did not change during differentiation of bNSCs (Supplementary Fig. S9A). These findings further support the notion that MMSET levels are regulated at the posttranscriptional level during differentiation. In support of this, we observed that the proteasome inhibitor MG-132 blocks the downregulation of Mmset type II in differentiating bNSCs (Supplementary Fig. S9B). Thus, it is likely that MMSET type II protein is targeted for proteolysis during differentiation of NPCs.

Discussion

In this study, we have shown that MMSET is highly expressed in neuroblastomas and is associated with aggressiveness. A relatively high level of MMSET was observed in human neuroblastoma SH-SY5Y cells and in mouse NPC, and this level decreased dramatically during differentiation presumably due to posttranscriptional regulation by proteolysis. Importantly, we have also shown that MMSET is required for proliferation of the neuroblastoma cell lines and bNSCs in tissue culture.

Although we have shown that MMSET is highly expressed in 75% of neuroblastomas in 2 independent cohorts; is associated with poor prognostic markers, poor survival, and progression; and is required for proliferation of neuroblastoma cells, MMSET expression does not qualify in the current study as an independent prognostic marker for poor survival of neuroblastoma patients. Additional studies including larger cohorts might qualify MMSET as an independent marker, providing extra prognostic significance to the currently used markers. Importantly, however, our results further qualify MMSET as a potential strong therapeutic target for the development of anticancer drugs. These results are also supported by searching the publicly available neuroblastoma microarray database on the Oncogenomics website.
(http://pob.abcc.ncifcrf.gov/cgi-bin/JK), where we found that high MMSET mRNA expression correlated with poor survival.

We have shown that MMSET is required for the proliferation of bNSCs and tumor cells in tissue culture; however, so far, it is not clear whether MMSET contributes to neuroblastoma tumor development or whether it is a marker of a cancer stem cell population, and the high expression observed in neuroblastosomas reflects a differentiation block of the cell of origin of neural crest (Supplementary Fig. S10C). Taken together our results presented by immature teratomas, in which MMSET was expressed mostly in primitive blastema-like tissue or neuroectoderm-derived primitive neuroepithelial cells (ref. 24 and Supplementary Fig. S10C). Taken together our results show that MMSET is expressed at high levels in the stem cell population, and the high expression observed in neuroblastomas (and other types of tumors) therefore might reflect a stem cell–like expression pattern in the cancer cells.

To address whether MMSET also could contribute to the development of neuroblastomas (i.e., be a driver of tumorigenesis), we ectopically expressed MMSET type II in bNSCs that subsequently was induced to undergo differentiation. However, as for the endogenous protein and as observed in treated tumors, differentiation signals led to a strong reduction in the ectopically expressed MMSET protein. This result could indicate that additional genetic changes are required for maintaining MMSET expression, and although a delay in differentiation was observed, that high levels of MMSET are not sufficient to induce a differentiation block of the neural stem cells.
The roles of the different isoforms of MMSET in normal and cancer cells are unclear. Because we do not have antibodies available that can distinguish between the various isoforms, it remains to be determined whether they are all highly expressed in neuroblastoma. Importantly, however, the full-length catalytically active MMSET type II has been shown to possess oncogenic potential (18), and although some myeloma cases have been found with only MMSET type I mRNA upregulated (32), most results suggest that MMSET type II is the driving oncogene.

In summary, we have found that high levels of MMSET expression are present in aggressive neuroblastomas with poor prognosis, that MMSET levels are high in the stem cell population, and that MMSET is required for proliferation of hNSC as well as neuroblastoma cells in culture. On the basis of these findings, we propose that MMSET is a potential marker for poor prognosis in neuroblastomas and a good candidate for the development of anticancer drugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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