MMSET expression in multiple tumors

The histone methyltransferase and putative oncoprotein

MMSET is overexpressed in a large variety of human tumors

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Statement of Translational Relevance

For many incurable human cancer types better prognostic markers and more effective treatments are required. The MMSET histone methyltransferase is frequently found translocated in multiple myelomas with poor prognosis. In this study we show that MMSET may play a much broader role in human cancer than previously anticipated. We demonstrate that MMSET is overexpressed in several types of human tumors, including carcinomas of the gastrointestinal tract and skin. Interestingly, in bladder cancer MMSET expression correlates with tumor aggressiveness. Taken together these results suggest that MMSET is a strong candidate target for the development of novel anti-cancer drugs and suggest that MMSET expression is a potential marker for tumor aggressiveness.
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Abstract

**Purpose:** MMSET is a histone lysine methyltransferase deregulated in a subgroup of multiple myelomas with the t(4;14)(p16;q32) translocation and poor prognosis. With the aim of understanding if MMSET can be involved in other types of cancer we investigated the expression of MMSET protein in different types of human tumors.

**Experimental design:** A monoclonal antibody against MMSET was developed and immunohistochemical staining of tissue microarrays (TMAs) containing a large number of tumor samples (n=3774) and corresponding normal tissues (n=904) was performed. Further validations of MMSET expression were carried out on independent, tumor-specific sets of TMAs for urinary bladder (n=1293) and colon cancer (n=1206) with corresponding clinico-pathological data and long-term follow-up.

**Results:** MMSET protein was highly expressed in different tumor types compared to normal counterparts. Particular frequent and/or high MMSET expression was found in carcinomas of the gastrointestinal tract (stomach, colon, anal canal), small cell lung carcinoma, tumors of the urinary bladder, female genitals, and skin. In bladder cancer, MMSET expression correlated with tumor aggressiveness. In contrast, MMSET expression was associated with good prognostic factors in colon cancer and was more pronounced in early stages of colon carcinogenesis (dysplasias) than in adenocarcinomas. However, colon cancer patients with high MMSET levels showed a worse 5-year survival.
Conclusions: Our data suggest that MMSET has a broader role in cancer than previously anticipated, and further analysis might qualify it as a prognostic marker and a target for the development of therapy against several types of cancer.
Introduction

MMSET/WHSC1/NSD2 is a SET domain containing histone lysine methyltransferase that can di- and trimethylate histone H3 at lysine 36 (H3K36) (1, 2). However, other specificities for MMSET have also been reported (3-5). The H3K36 methylation mark is present in transcriptionally active genes (6) presumably contributing to repression of inappropriate transcription inside transcribed genes (7, 8). In addition to the SET domain, MMSET also contains other conserved domains including plant homeodomain (PHD) zinc fingers, a high mobility group box (HMG) domain, and proline-tryptophane rich (PWWP) domains (Fig 1A), found in proteins with DNA and chromatin binding activities (9-12). The MMSET gene is located at chromosome 4p16.3 and undergoes alternative splicing (13-15) resulting in several protein isoforms: MMSET type I, MMSET type II, and RE-IIBP (Fig. 1A). Northern blotting has revealed that MMSET mRNA is expressed in highly proliferating embryonic tissues while primarily in adult thymus and testis (3, 13).

MMSET is also known as nuclear receptor SET domain (NSD)2 and belongs to a family of NSD proteins (1-3) (16). These proteins are related to S. cerevisiae Set2 and they all posses H3K36 histone methyltransferase activity (1, 2, 17, 18). Another name for MMSET is Wolf-Hirschhorn syndrome candidate 1 (WHSC1), because it maps to the Wolf-Hirschhorn syndrome (WHS) critical region at 4p16.3 (14). This region is invariably lost in all cases of WHS, a congenital syndrome characterized by several somatic defects and mental retardation. MMSET is involved in the reciprocal t(4;14)(p16;q32) translocation in a subgroup of multiple myeloma (15-20% of cases) that
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is associated with poor prognosis (13, 19-22). High expression of MMSET appears to be involved in myelomagenesis, as the t(4;14) translocation has been detected at a significantly lower frequency in patients with the benign condition, monoclonal gammopathy of undetermined significance (MGUS) (21, 23, 24). Recent data have shown that MMSET expression is essential for growth of myeloma cell lines in tissue culture and in xenografts, indicating that MMSET contributes to tumor maintenance \textit{in vivo} (4, 25, 26). However, a direct contribution of aberrantly expressed MMSET to tumorigenesis remains to be shown.

Recent studies have also described high levels of MMSET mRNA in hepatocellular carcinoma (27) and leukemia (3). Moreover, a search in the Oncomine Cancer Microarray database (28) has shown that MMSET mRNA expression is upregulated in 15/40 tumor types compared to their normal tissue counterparts. For several of these cancers, MMSET mRNA expression appeared to be associated with tumor aggressiveness (28). So far, no studies have reported on the expression of MMSET protein in different human tumors.

In this study, we have addressed MMSET protein expression in multiple human tumor types. We found that MMSET expression is frequently detected in many different cancers in comparison to the corresponding normal tissues. Particular frequent and/or high MMSET protein expression was found in carcinomas of the gastrointestinal (GI) tract (esophagus, stomach, colon, anal canal), small cell lung carcinoma (SCLC), and tumors of the urinary bladder, female genitals, and skin.
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Materials and Methods

Tissue culture

Human osteosarcoma U2OS cells were cultured in a humidified incubator at 37°C with 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) with 10% FCS (Hyclone), 100 μg/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco).

Production of a MMSET monoclonal antibody and applicability on formalin-fixed cells.

The MMSET antigen fragment (corresponding to amino acids 1-119 of MMSET type I and II, Fig. 1A) was amplified by PCR using forward primer: 5’-CGGGATCCATGGAATTTAGCATCA-3’ and reverse primer: 5’-CGGGATCCATTTTTGATAGGGGTAGT-3’ (including BamHI overhangs) and cloned into the TA cloning vector pCR2.1 (Invitrogen). The fragment was subcloned into pGEX-20T (a derivative of pGEX2T, Amersham Pharmacia Biotech) by BamHI digestion and verified by sequencing. It was expressed in BL21 cells (Invitrogen) by induction with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 3 hours and subsequently purified with Glutathione Sepharose beads. The purified antigen was injected subcutaneously into mice and after 6 months hybridomas were made according to standard procedures. The monoclonal antibody (mAb), purified from hybridoma clone 9A6, was effectively used for immunohistochemistry (IHC). The specificity and applicability of 9A6 mAb in immunostainings of formalin-fixed, paraffin-embedded (FFPE) cells were first verified in U2OS cells expressing either pGIPZ-MMSET I+II shRNA (Open Biosystems; clone ID: V2LHS_201908; TGCTGTGGACAGTGGCAGCGCAAGTCACCTTTCCTATTTAGTGAAGCCACA

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GATGTAATAGAGAAAGGTGAACCTTGCTTGCCTACTGCCTCGGA) or empty pGIPZ.

*Immunoblotting*

Protein extracts for immunoblots were obtained using high-salt lysis buffer (50 mM Tris, pH 7.2, 300 mM NaCl, 0.5%, IGEPAL CA-630, 1 mM DTT, 1 mM EDTA, 1 μg/ml Leupeptin, 1 μg/ml Aprotinin and 1mM PMSF) and sonication (Branson Digital Sonifier, 3x 5 sec, 20%, 0°C). Immunoblotting was performed according to standard protocols. Antibodies used: 9A6 mAb (1:4; hybridoma supernatant), β-TUBULIN (Santa Cruz, sc-9104, 1:10000)

*Tissue microarrays and staining with MMSET antibody*

MMSET protein expression was analyzed by comprehensive IHC screenings of tissue microarrays (TMA) containing a total of 3774 FFPE archival samples (diameter 0.6 mm) from many different human tumors and 904 corresponding controls from normal tissues (cohort 1) (29). Further validation of MMSET expression patterns in colorectal adenocarcinomas and urinary bladder tumors was carried out on independent, tumor-specific sets of TMA (cohort 2: colorectal adenocarcinoma, n=1206; cohort 3: urinary bladder tumors, n=1293) (29). Corresponding clinico-pathological data and long-term follow-up were available for all patients in cohort 2 and 3. All these archival samples (provided by RS and GS) were derived from tissues previously collected for routine diagnostic procedures performed at the Dept. of Pathology, University Medical Center Hamburg-Eppendorf, in accordance with the principles of the “Ethik-Kommission der
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Ärztekammer, Hamburg”. The collection and TMA-based screenings of human tumor samples were in compliance 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 the treatment of patients.

Immunostaining for MMSET expression was performed on de-paraffinized TMA sections by initially retrieving antigens in Tris-EDTA-citrate (TEC, pH 7.8) buffer in an autoclave for 5 min at full temperature. After cooling down the slides, blocking of endogenous peroxidase activity was performed according to the protocol of Envision+ mouse kit (Dako). The TMA samples were then incubated with MMSET 9A6 mAb (0.5 μg/μl, diluted 1:150) in 1% BSA/TBS buffer at 4°C overnight. The reaction was visualized by sequentially incubating with secondary antibody (Envision+ Linker-Mouse, Dako), horseradish peroxidase, and diaminobenzidine (DAB, Dako; according to manufacturer’s instructions). Sections were counterstained with Mayer’s hematoxylin for 1 min.

The MMSET immunostainings were analyzed by a pathologist (ESR) blind to clinico-pathological data and were scored as follows:

0 (negative): No nuclear staining
1 (weak): < 10% positive cells (and weak nuclear staining intensity)
2 (moderate): between 10-50% positive cells (and moderate nuclear staining intensity)
3 (strong): > 50% positive cells (and strong nuclear staining intensity)
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The terms in brackets indicate that in the vast majority of the samples, a correlation was observed between the number of positive cells and the intensity of the nuclear staining. A minimum of 100 relevant cells was counted. As a reference value of score 2, the positive staining of germinal centers in lymph nodes with follicular hyperplasia was used. Omission and substitution of 9A6 mAb with unspecific Ig was utilized as negative control. 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.

Biopsies from multiple myeloma patients

A series of 39 FFPE bone marrow biopsies from patients diagnosed with multiple myeloma (provided by ER, Dept. of Pathology, Rigshospitalet, University of Copenhagen) were immunostained with the 9A6 mAb. These archival samples were collected as part of the routine diagnostic procedures according to the guidelines of the Danish National Board of Health.

Statistics

All statistic analyses were performed using R. Fisher’s exact test (for total counts >500, p-values were estimated using Monte Carlo simulations) and Kaplan-Meier log-rank test were used. A p<0.05 was considered significant.
Results

Development of a MMSET specific monoclonal antibody

The GST-tagged antigen and the correspondingly generated MMSET mAb (9A6) are shown in Fig. 1A. The 9A6 mAb recognizes the protein isotypes MMSET type I and II (but not RE-IIBP) and the specificity of the antibody was demonstrated by immunoblotting (Fig. 1B). U2OS cells were transduced either with a lentivirus expressing a shRNA knocking down MMSET I+II or with control virus for 48 hours and subsequently selected with puromycin for 48 hours. The cells were harvested, lysed, and resolved by SDS-PAGE followed by immunoblotting (Fig. 1B). U2OS cells were chosen for this test due to a relatively high expression of endogenous MMSET type I and II protein compared to many other cell lines tested (not shown) but a lower expression than in t(4;14)+ myeloma cells lines (Supplementary S1A). Furthermore, the 9A6 mAb was found to be effective in immunostainings when applied to the FFPE sections of U2OS cells (Fig. 1C and Supplementary Fig. S1B). MMSET was located in the nucleus of cells infected with control virus and absent in the majority of cells infected with MMSET I+II shRNA (Fig. 1C). All together, these results show that 9A6 specifically recognizes MMSET type I and II in FFPE cells.

Expression levels of MMSET protein in different human tumor types

Since a subgroup of 15-20% of myelomas displays overexpression of MMSET mRNA due to the t(4;14)(p16;q32) translocation (21, 22), we investigated MMSET protein expression in a series of bone marrow biopsies from patients with multiple myeloma. IHC showed that 13/39 (33%) myelomas displayed MMSET protein levels correlating...
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with the presence of the t(4;14) translocation detected by fluorescence in situ hybridization (FISH, Fig. 2, Supplementary Fig. S1C, and Supplementary table I). In hyperplastic lymph nodes, used as a non-neoplastic control (n=33), MMSET expression was detected in lymphocytes of the germinal centers and scattered lymphocytes of the marginal zone, but not in the plasma cells (Fisher’s exact test, $p=5.3\times10^{-4}$; Supplementary Fig. S1D). Therefore, IHC for MMSET protein confirmed that the t(4;14)$^+$ subgroup of multiple myeloma expresses high levels of MMSET compared to normal plasma cells.

We then determined the expression level of MMSET in 3774 samples from different types of human tumors and 904 samples from corresponding normal tissues. Interestingly, high and/or frequent MMSET expression was detected in many different cancer types, in contrast to most of the corresponding normal tissues or most benign tumors that showed no MMSET expression (Table I).

Among non-neoplastic tissues, MMSET was expressed by thymocytes of normal or hyperplastic thymus (8/8: 100%), which is consistent with previous studies showing MMSET mRNA expression in normal adult thymus (3, 13). In addition, 11/15 (73%) hyperplastic tonsil samples expressed MMSET at low levels in the lymphatic germinal centers and in scattered activated lymphocytes, similarly to what was observed in hyperplastic lymph nodes (Supplementary Fig. S1D). Finally, a few scattered, weakly positive cells were sporadically observed in the basal layer of the esophageal squamous epithelium (3/13: 23%), in epithelial cells of gastric (1/21: 5%) and anal (2/20: 10%) mucosa, and in the endometrium (3/33: 9%).
Importantly, MMSET protein expression was primarily present in malignant tumors (Table I). Consistent with this notion, mucoepidermoid carcinoma, the only malignant tumor of the salivary glands represented in the TMA, was also the only one to show some MMSET expression (12/39: 31%) in comparison to normal parotis tissue and benign salivary gland tumors, such as Whartin’s tumor, pleomorphic adenoma, and basal cell adenoma (Table I). Frequent and/or high MMSET expression was detected in carcinomas of the lower respiratory tract (Fig. 3A), and GI tract (esophagus, stomach, colon, and anal canal; Fig. 3B, C and Table I). In addition, high expression was observed in urinary bladder tumors (Table I), and in a fraction of serous and endometroid carcinomas of ovary and corpus uteri as well as in cervix carcinomas (Supplementary Fig. S2). Also, some skin tumors displayed MMSET expression (Supplementary Fig. S3).

The majority of testicular seminomas and all non-seminomatous germ cell tumors were MMSET-negative, while 14/53 teratomas (26%) showed significant expression, though mostly confined to blastema-like component or primitive neuroepithelial structures present in immature teratomas (Table 1). Few samples representing the various subtypes of breast carcinoma showed some, but mostly weak MMSET expression (Table 1). Mammary phylloides tumors were all negative, whereas significant expression was detected in breast carcinosarcomas (10/35: 29%) and stromal sarcomas (5/12: 42%). Approximately 33% of malignant fibrous histiocytomas (n=24) and 33% of liposarcomas (n=12) displayed weak or moderate MMSET-positive immunostaining. The general TMA screening also revealed several cancer types with no or minimal MMSET
expression (Table I), suggesting that MMSET is unlikely to play an important role in the maintenance of these tumors.

**Tumors of the lower respiratory tract**

Among lung tumors, the highest frequency and intensity of MMSET expression was detected in SCLCs (8/15: 53%; Fig. 3A, left). In contrast, MMSET was not detected in normal bronchus (0/16 samples; Fisher’s exact test, \( p=8.2 \times 10^{-4} \)). MMSET was also expressed in non-small cell lung carcinomas (NSCLCs), such as squamous carcinoma, adenocarcinoma, bronchioloalveolar carcinoma (BAC), and large cell neuroendocrine carcinoma (LCNEC), as well as in malignant mesothelioma (between 9-28% of samples), whereas no expression was observed in normal lung parenchyma and pleura (0/40). Representative MMSET immunostainings in squamous carcinoma, adenocarcinoma, LCNEC, and SCLC are shown (Fig. 3A, right). Interestingly, BAC, a highly differentiated subtype of adenocarcinoma with non-invasive growth along pre-existing alveolar septa (30) showed less frequent MMSET expression than other subtypes of adenocarcinoma (Table I).

**Tumors of the gastrointestinal tract**

Among the tumors of the GI tract, frequent MMSET expression was detected in squamous carcinomas (21/54: 39%) and adenocarcinomas (31/55: 56%) of the esophagus (Table 1) and adenocarcinomas of the stomach with a significant preponderance for the intestinal subtype (33/54: 61%; Fisher’s exact test, \( p=1.0 \times 10^{-4} \); Fig. 3B, left). In comparison 1/21 (5%) normal stomach mucosa sample was weakly positive (Fig. 3B,
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Representative examples of the MMSET immunostainings in gastric normal mucosa and intestinal adenocarcinoma are shown in Fig. 3B (right). The diagram in Fig. 3C (left) illustrates the distribution of MMSET expression among the anal squamous carcinomas where 9/16 (56%) samples were positive for MMSET expression. Only 2/20 (10%) normal mucosa samples from the anal canal were weakly positive for MMSET staining (Fisher’s exact test, \( p=0.010 \)). Representative images of MMSET immunostainings in normal mucosa and basaloid squamous carcinoma of the anal canal are shown in Fig. 3C (right).

**MMSET expression in colon tumors**

MMSET expression was found to be high in colorectal adenocarcinomas and premalignant dysplasia (Fig. 4A, left). The 39 samples from the normal mucosa all stained negative for MMSET, while the expression frequency of MMSET was 37/51 (73%) in low-grade dysplasia (Fisher’s exact test, \( p=4.8\times10^{-13} \)), 34/37 (92%) in high-grade dysplasia (Fisher’s exact test, \( p=1.7\times10^{-18} \)), and 56/114 (49%) in adenocarcinoma (Fisher’s exact test, \( p=5.5\times10^{-8} \)). Representative images of MMSET stainings of normal mucosa, low- and high-grade dysplasia, and adenocarcinoma are shown (Fig. 4A, right).

To validate these observations, we determined the expression of MMSET immunostainings in another cohort of colorectal adenocarcinomas (cohort 2, n=1206) for which corresponding clinico-pathological data and long-term follow-up were available (Supplementary table II), thus allowing clinical correlation with MMSET protein expression. MMSET expression was detected in 643/1206 (53%) adenocarcinomas versus no expression in normal colon mucosa (0/13: 0%; Fisher’s exact test, \( p=2.1\times10^{-3} \);
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Fig. 4B) and was comparable to MMSET expression in cohort 1 (compare Fig. 4A and Fig. 4B).

We found a significant difference in MMSET expression in the different stages of the tumor where pT1 differed significantly from pT3 and pT4 (Fig. 4C, left; Fisher’s exact test, $p=0.023$ and $p=1.5\times10^{-4}$, respectively). However, surprisingly, our results showed that MMSET expression decreases with increasing stage. In agreement with this, we also observed a statistically significant inverse correlation between MMSET expression and pN (Fig. 4C, middle; Fisher’s exact test; pN0 vs. pN1, $p=7.0\times10^{-3}$; pN0 vs. pN2, $p=3.3\times10^{-5}$). Furthermore, MMSET expression differed according to whether the tumor margin was pushing or infiltrating (Fig. 4C, right, Fisher’s exact test, $p=4.1\times10^{-5}$). From these data, we conclude that MMSET expression is inversely correlating with known negative prognostic factors (stage, pN, invasive margin) for colorectal adenocarcinomas.

In contrast, we observed a negative correlation between MMSET expression and survival (Fig. 4D; log-rank test, $p=7.4\times10^{-6}$). However, the difference in survival according to MMSET expression was first visible approximately 4 years after diagnosis. In agreement with this, a negative correlation between MMSET expression and survival was determined by multivariate Cox regression analysis (corrected for other prognostic factors such as stage, pN, grade, pushing vs. infiltrating margin, age, tumor diameter, presence vs. absence of peritumoral lymphocytes, presence vs. absence of vascular invasion).
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The clinical data for the colorectal adenocarcinomas also included age, sex, tumor diameter, localization, and differentiation grade, presence vs. absence of peritumoral lymphocytes, presence vs. absence of vascular invasion. None of these features showed a significant correlation with MMSET expression (data not shown).

Based on these results, we suggest that MMSET expression could play an important role in progression into the early stages of colorectal carcinogenesis (dysplasia). However, 4 years after diagnosis, MMSET expression is an independent negative prognostic marker.

**MMSET expression in bladder cancer**

MMSET expression is also high in tumors of the urinary bladder (Fig. 5A, left). No MMSET expression was detected in 20 normal urothelial mucosa samples while MMSET expression was observed in 25/59 (42%) of non-invasive urothelial tumors (pTa; Fisher’s exact test, \( p=3.8 \times 10^{-3} \)) and in 28/55 (51%) of invasive urothelial tumors (pT2-pT4; Fisher’s exact test, \( p=4.8 \times 10^{-4} \)). Very strong MMSET expression was observed in 4/8 samples from small cell carcinomas (SCC; 50%; Fisher’s exact test, \( p=3.4 \times 10^{-3} \)). Interestingly, we noticed that the 6 invasive urothelial tumors with strong MMSET expression (score 3) all were high-grade G3 tumors. Together with very strong expression in half of the SCC, these data could suggest a role for MMSET levels in determining the phenotype of urothelial tumors. Representative images of MMSET stainings of normal urothelial mucosa, low- and high-grade urothelial tumors, and SCC are shown (Fig. 5A, right). To validate the findings of cohort 1 in an independent and larger cohort, we analyzed the expression of MMSET in 1293 urothelial tumors (cohort
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3), for which corresponding clinico-pathological data and long-term follow-up were available (Supplementary table III). MMSET protein was detected in 520 of these tumors (40%, Fig. 5B). When stratified into different histological tumor types (including adenocarcinomas, n=14; sarcomatous carcinoma, n=13; urothelial carcinoma, n=1154; squamous carcinoma, n=46; SCC, n=20), SCC had stronger and more frequent expression pattern of MMSET than the other histological tumor types (75% positive for MMSET protein, Fig. 5B). The other tumor histotypes did not differ significantly from each other. The MMSET expression pattern in the urothelial tumors in cohort 3 was comparable to cohort 1 (compare Fig. 5A and Fig. 5B). The MMSET expression pattern of SCC in cohort 3 differed from cohort 1 (compare Fig. 5A and Fig. 5B), however both cohorts contained relatively few samples of SCCs, and in our further analysis the entire pool of urothelial tumors represented in cohort 3 was therefore included.

Interestingly, we found a strong positive correlation between MMSET expression and the grade of the tumor in agreement with the strong MMSET expression in G3-tumors in cohort 1 (Fig. 5C, upper left; Fisher’s exact test; G1 vs. G2, \( p=1.3\times10^{-5} \); G1 vs. G3, \( p=1.0\times10^{-6} \)). In agreement with this finding, a positive correlation was also found between MMSET expression and the stage of the tumor (Fig. 5C, upper right; Fisher’s exact test, pTa vs. pT1, \( p=1.0\times10^{-6} \); pTa vs. pT2-pT4, \( p=1.0\times10^{-6} \)). The same trend was observed for cohort 1 (Fig. 5A). Furthermore, age also correlated positively with MMSET expression (Fig. 5C, lower left; Fisher’s exact test, \( p=4.6\times10^{-3} \)) and MMSET expression differed according to whether the growth of the tumor was solid or papillary (Fig. 5C, lower right; Fisher’s exact test, \( p=7.8\times10^{-3} \)). In addition, MMSET expression
MMSET expression in multiple tumors correlated positively with progression to more aggressive tumor stages (Fig. 5D; log-rank test, $p=6.3\times10^{-4}$).

The clinical data for the bladder cancer patients also included survival, sex, several samples from a patient over time, unicentric versus multicentric tumors, and smoking status. However, none of these parameters showed a statistically significant correlation with MMSET expression (data not shown).

Taken together, our results indicate that MMSET plays a role in development of aggressive urinary bladder cancer.
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Discussion

In this study, we have shown that the MMSET protein is frequently expressed in many different human cancer types in comparison to normal counterparts and most benign tumors. This issue has not previously been addressed at the protein level in different cancer types. MMSET protein expression in cancer has only been demonstrated in myeloma cell lines (4) and in glioblastoma in comparison to normal brain cortex (31). However, in our screening, we only detected a weak MMSET expression in 4/26 (15%) oligodendrogliomas and no MMSET expression was observed in astrocytomas (n=44) ranging from low- to high-grade (glioblastomas, Table I). In multiple myeloma, we detected MMSET expression in 33% of samples that highly correlated with the presence of the t(4;14) translocation (present in 38%). Due to an MMSET intragenic translocation breakpoint in approximately 33% of the t(4;14)+ myeloma cases (19, 23, 32, 33), the 9A6 mAb (recognizing the N-terminal part of MMSET) is not able to detect overexpressed truncated MMSET in these myeloma cases. In line with this, we found 3/10 t(4;14)+ myelomas where the 9A6 mAb did not detect the MMSET protein (Supplementary Table I). The detection of 38% t(4;14)+ myelomas should be compared to the observation that the t(4;14) translocation and increased levels of MMSET mRNA are normally detected in 15-20% of multiple myelomas (21, 22). The overrepresentation of the t(4;14)+ subgroup could be due to the small number of myelomas, as they were randomly included in the study. To understand the frequency of MMSET overexpression in multiple myeloma and the correlation with the t(4;14) translocation it would be interesting to screen a larger panel of primary tumors. Here, a combination of mAbs recognizing all the different
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MMSET protein isoforms including the N-terminal truncated ones (resulting from the t(4;14) translocation) should be used.

In agreement with our study, high MMSET mRNA levels have been observed in cancers from bladder, colon, esophagus, liver, lung, and ovary in comparison to normal control tissue (27, 28). However, we also observed high MMSET protein levels in malignant tumors from salivary glands, stomach, cervix uteri, corpus uteri, and skin. These differences could be explained by the fact that the approaches were different and that mRNA and protein are not always comparable and can be regulated in different ways. In fact, our unpublished results suggest that MMSET is regulated at posttranslational levels during differentiation, and that increased MMSET protein levels therefore could be a result of the stability of the MMSET protein.

In urinary bladder cancer, MMSET protein was detected in 40% of the cases and was associated with aggressiveness: expression levels correlated with poor prognostic markers (stage, grade, age and type of tumor growth), and progression. In line with this, a correlation between the grade of the urinary bladder cancer and MMSET mRNA expression has also been described (28).

Interestingly, we have demonstrated that the expression levels of MMSET in colon tumors differed from what we observed for other tumor types in that the frequency of MMSET protein expression was highest in the benign stages (low- and high-grade dysplasia, 73% and 92%, respectively) in comparison to colorectal adenocarcinoma
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(cohort 1 and 2, 49 and 53%, respectively) and MMSET expression correlated inversely with poor prognostic markers (stage, pN, and type of invasive margin). However, approximately 4 years after diagnosis, MMSET protein expression correlated with poor survival. Based on these results, we are considering whether MMSET expression is important for progression into colon dysplasia and maybe further into colorectal adenocarcinoma. Hereafter, in the early and less aggressive forms of adenocarcinoma, there seems to be a selection against MMSET expression. Potentially, stronger and more relevant genetic alterations or “tumor hits” take over making MMSET expression dispensable. After 4 years, the expression of MMSET in combination with acquired genetic aberrations obtained along with progression into more aggressive stages, will lead to poor survival. Interestingly, a similar observation has been described for B-RAF in benign nevus and malignant melanoma, which both arise from melanocytes. B-RAF is highly and frequently expressed due to mutations in the \( B-RAF \) gene. High expression of B-RAF in benign nevus mostly leads to senescence whereas in melanoma, high B-RAF expression leads to a poor outcome (34-36).

It would be of great interest to investigate the role of MMSET in more cancer types where MMSET is highly and/or frequently expressed. We are currently investigating the expression of MMSET in diffuse large B-cell lymphoma (DLBCL), as MMSET expression was detected in 89% of the DLBCL samples (n=9, Table I). The mechanisms leading to high levels of MMSET expression in the different types of tumors remain to be elucidated. Moreover, it remains to be determined whether aberrant MMSET expression is directly contributing to tumor development or is a marker of transformed cells. Finally,
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the role of the histone methyltransferase activity in the putative oncogenic function of MMSET remains to be established. Recently, the global level of H3K36 methylation was shown to correlate with MMSET expression in a panel of myeloma cell lines (37). Similarly, we observed a relatively high level of H3K36 dimethylation in t(4;14)⁺ versus t(4;14)⁻ myeloma cell lines and no significant difference in H3K36 trimethylation level was observed (Supplementary Fig. S1A). However, our studies show that the expression levels of MMSET do not correlate with global levels of H3K36 di or tri-methylation in the U2OS cell line and in primary myeloma samples (Supplementary Fig. S4 and Supplementary table I), which could suggest that MMSET might contribute to tumorigenesis through the modulation of histone levels of specific genes.

In summary, the current study demonstrates that MMSET is highly expressed in a large number of different types of tumors, and the protein might therefore have a much broader role in transformation than previously anticipated. MMSET may be a future prognostic marker of several tumor types and is a new potential therapeutic target in treatment of different cancer types.
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Figure Legends

Figure 1. Generation of a MMSET monoclonal antibody.

A) The different MMSET protein isotypes are shown with their predicted conserved domains. C5HCH: NSD specific zinc finger, HMG: high mobility group box, NLS: nuclear localization signal, PHD: plant homeo domain zinc finger, PWWP: proline tryptophane rich domain, SET: Suppressor of variegation, Enhancer of zeste, and Trithorax. The antigen used to raise the 9A6 mAb is illustrated as a blue line. B) Immunoblotting of U2OS cells infected with shRNA constructs targeting MMSET type I+II or empty shRNA was performed with the 9A6 mAb. Immunoblotting with β-TUBULIN was included as loading control. C) The cells described in B were embedded in paraffin and immunostained with 9A6 mAb. 40x magnification. (Haematoxylin-eosin staining and negative control can be seen in Supplementary Fig. S1B). a.a.: amino acids.

Figure 2. MMSET protein expression in multiple myeloma.

Expression pattern of MMSET protein in a panel of 39 multiple myelomas and in normal plasma cells in 33 hyperplastic lymph nodes is illustrated in the diagram. The expression level is distributed into negative (0), weak (1), moderate (2), and strong (3) expression. Representative MMSET immunostainings of 2 multiple myelomas are shown (MM 31 and 39 in Supplementary table I). 20x magnification. MM: multiple myeloma, N PC: normal plasma cells.

Figure 3. MMSET expression pattern in lung, stomach, and anal tumors.
MMSET expression in multiple tumors

Expression pattern of MMSET in tumors of the lung (A), stomach (B), and anal canal (C) in comparison to the normal control tissue is illustrated (left). Representative MMSET immunostainings from the TMA are shown to the right. If no \( p \)-value is noted, no significant difference in MMSET expression was found between the tumor and the corresponding normal tissue. 10x and 20x magnifications. ACa: adenocarcinoma, BAC: bronchioloalveolar carcinoma, LCNEC: large cell neuroendocrine carcinoma, N: normal, SCLC: small cell lung carcinoma, Sq Ca: squamous carcinoma.

**Figure 4. MMSET expression in colon cancer.**

A) MMSET expression in colorectal normal mucosa, dysplasia, and adenocarcinomas from cohort 1 (left). Representative MMSET immunostainings from the TMA are shown to the right. 10x and 20x magnifications. B) MMSET expression pattern in colon samples from cohort 2. C) MMSET expression in the different stages (pT1-4) (left), according to lymph node status (middle), and in pushing versus infiltrating margin (right) in colorectal adenocarcinomas from cohort 2. D) Kaplan-Meier plot showing 5 year-survival of patients with colorectal adenocarcinomas from cohort 2. ACa: adenocarcinoma, N: normal.

**Figure 5. MMSET expression in urinary bladder cancer.**

A) MMSET protein expression in urinary bladder tumors and normal urothelial mucosa from cohort 1 (left). Representative immunostainings of MMSET are shown to the right. 10x, 20x, and 40x magnifications. B) MMSET expression in the pool of tumors and in the different histological tumor types in cohort 3. C) MMSET expression in the different
MMSET expression in multiple tumors

grades (G1-3; upper left), in the different stages of the tumor (pTa, pT1, and pT2-4; upper right), in different age groups (lower left), and in solid versus papillary growth pattern (lower right) in urothelial tumors from cohort 3. D) Kaplan-Meier plot showing probability of progression of the tumor to higher stages over a 5 year-period. ACa: adenocarcinoma, Ca: carcinoma, N: normal, SCC: small cell carcinoma; Sq Ca: squamous carcinoma.
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References

9. Love JJ, Li X, Chung J, Dyson HJ, Wright PE. The LEF-1 high-mobility group domain undergoes a disorder-to-order transition upon formation of a complex with cognate DNA. Biochemistry 2004;43: 8725-34.
MMSET expression in multiple tumors

## Table 1. Quantification of MMSET immunostainings on TMA

<table>
<thead>
<tr>
<th>Non-neoplastic control tissue / Tumor type</th>
<th>Nr. of positive samples / total samples</th>
<th>%</th>
<th>Intensity of nuclear immunostaining</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal lip/oral mucosa</td>
<td>0/17</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral Squamous Ca</td>
<td>11/52</td>
<td>21.2%</td>
<td>weak</td>
<td></td>
</tr>
<tr>
<td>Esophagus, normal mucosa</td>
<td>3/13</td>
<td>23.1%</td>
<td>Weak</td>
<td>Scattered basal cells in epithelium</td>
</tr>
<tr>
<td>Esophagus – Squamous Ca</td>
<td>21/54</td>
<td>38.9%</td>
<td>6 weak, 9 moderate, 6 strong</td>
<td></td>
</tr>
<tr>
<td>Esophagus – Adenocarcinoma</td>
<td>31/55</td>
<td>56.4%</td>
<td>9 weak, 22 moderate</td>
<td></td>
</tr>
<tr>
<td>Stomach, normal mucosa</td>
<td>1/21</td>
<td>4.8%</td>
<td>weak</td>
<td></td>
</tr>
<tr>
<td>Stomach Adenocarcinoma, Intestinal type</td>
<td>33/54</td>
<td>61.1%</td>
<td>12 weak, 12 moderate, 9 strong</td>
<td></td>
</tr>
<tr>
<td>Stomach Adenocarcinoma, Diffuse type</td>
<td>14/47</td>
<td>29.8%</td>
<td>11 weak, 3 moderate</td>
<td></td>
</tr>
<tr>
<td>Duodenum, normal mucosa</td>
<td>0/23</td>
<td>0%</td>
<td>15 samples from mucosa, 8 only Brunners glands</td>
<td></td>
</tr>
<tr>
<td>Duodenal Adenocarcinoma</td>
<td>7/20</td>
<td>35%</td>
<td>3 weak, 4 moderate</td>
<td></td>
</tr>
<tr>
<td>Ileum, normal mucosa</td>
<td>0/8</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinoid Tumor (Intestinal)</td>
<td>3/35</td>
<td>8.6%</td>
<td>moderate</td>
<td></td>
</tr>
<tr>
<td>Colorectal normal mucosa</td>
<td>0/39</td>
<td>0%</td>
<td></td>
<td>23 from colon, 8 from appendix, 8 from rectum</td>
</tr>
<tr>
<td>Colon Dysplasia, low-grade</td>
<td>37/51</td>
<td>72.5%</td>
<td>22 weak, 12 moderate, 3 strong</td>
<td></td>
</tr>
<tr>
<td>Colon Dysplasia, high-grade</td>
<td>34/37</td>
<td>91.9%</td>
<td>9 weak, 13 moderate, 12 strong</td>
<td></td>
</tr>
<tr>
<td>Colon Adenocarcinoma</td>
<td>56/114</td>
<td>49.1%</td>
<td>27 weak, 9 moderate, 20 strong</td>
<td></td>
</tr>
<tr>
<td>Anal canal, normal mucosa</td>
<td>2/20</td>
<td>10.0%</td>
<td>weak</td>
<td>13 from skin of anal canal, 7 from transitional mucosa</td>
</tr>
<tr>
<td>Anal Squamous Ca</td>
<td>9/16</td>
<td>56.3%</td>
<td>4 weak, 3 moderate, 2 strong</td>
<td>Includes Basaloid Squamous Ca</td>
</tr>
<tr>
<td>Gallbladder, normal mucosa</td>
<td>0/18</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallbladder Ca</td>
<td>7/27</td>
<td>25.9%</td>
<td>weak</td>
<td></td>
</tr>
<tr>
<td>Liver, non-neoplastic</td>
<td>0/8</td>
<td>0%</td>
<td></td>
<td>Histological heterogeneous pattern (trabecular, solid and pseudoglandular)</td>
</tr>
<tr>
<td>Hepatocellular Ca</td>
<td>12/52</td>
<td>23.1%</td>
<td>5 weak, 7 moderate</td>
<td></td>
</tr>
<tr>
<td>Pancreas, non-neoplastic</td>
<td>0/18</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreas, Ductal adenocarcinoma</td>
<td>11/50</td>
<td>22.0%</td>
<td>6 weak, 5 moderate</td>
<td></td>
</tr>
<tr>
<td>Pancreas, Papillary adenocarcinoma</td>
<td>5/25</td>
<td>20.0%</td>
<td>weak (single cells)</td>
<td></td>
</tr>
<tr>
<td>Pancreas, Neuroendocrine tumor</td>
<td>3/19</td>
<td>15.8%</td>
<td>Weak</td>
<td></td>
</tr>
</tbody>
</table>
MMSET expression in multiple tumors

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Cases</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salivary glands, non-neoplastic</td>
<td>0/42</td>
<td>0%</td>
</tr>
<tr>
<td>Parotis Gland - Whartin Tumor</td>
<td>0/54</td>
<td>0%</td>
</tr>
<tr>
<td>Parotis Gland, Pleomorphic Adenoma</td>
<td>0/60</td>
<td>0%</td>
</tr>
<tr>
<td>Salivary Glands, Basal Cell Adenoma</td>
<td>0/37</td>
<td>0%</td>
</tr>
<tr>
<td>Salivary glands, Mucoepidermoid Ca</td>
<td>12/39</td>
<td>30.8%</td>
</tr>
<tr>
<td>Thyroid, non-neoplastic</td>
<td>0/10</td>
<td>0%</td>
</tr>
<tr>
<td>Thyroid – Adenoma</td>
<td>0/63</td>
<td>0%</td>
</tr>
<tr>
<td>Thyroid – Follicular Ca</td>
<td>0/46</td>
<td>0%</td>
</tr>
<tr>
<td>Thyroid – Medullary Ca</td>
<td>0/23</td>
<td>0%</td>
</tr>
<tr>
<td>Thyroid – Anaplastic Ca</td>
<td>0/3</td>
<td>0%</td>
</tr>
<tr>
<td>Thyroid – Papillary Ca</td>
<td>3/50</td>
<td>6.0%</td>
</tr>
<tr>
<td>Adrenal gland, non-neoplastic</td>
<td>0/12</td>
<td>0%</td>
</tr>
<tr>
<td>Adrenal Cortical Adenoma</td>
<td>0/20</td>
<td>0%</td>
</tr>
<tr>
<td>Adrenal Cortical Ca</td>
<td>0/8</td>
<td>0%</td>
</tr>
<tr>
<td>Pheochromocytoma (adrenal paraganglioma)</td>
<td>3/60</td>
<td>0%</td>
</tr>
<tr>
<td>Paraganglioma</td>
<td>5/35</td>
<td>14.3%</td>
</tr>
<tr>
<td>Larynx, normal mucosa</td>
<td>0/1</td>
<td>0%</td>
</tr>
<tr>
<td>Larynx- Squamous Ca</td>
<td>7/52</td>
<td>13.5%</td>
</tr>
<tr>
<td>Bronchus, non-neoplastic</td>
<td>0/16</td>
<td>0%</td>
</tr>
<tr>
<td>Lung parenchyma/pleura, non-neoplastic</td>
<td>0/40</td>
<td>0%</td>
</tr>
<tr>
<td>Lung – Squamous Ca</td>
<td>16/67</td>
<td>23.9%</td>
</tr>
<tr>
<td>Lung – Bronchioloalveolar Ca</td>
<td>1/11</td>
<td>9.1%</td>
</tr>
<tr>
<td>Lung – Adenocarcinoma</td>
<td>26/94</td>
<td>27.7%</td>
</tr>
<tr>
<td>Lung - NSCLC (unspecif. subtype)</td>
<td>3/8</td>
<td>37.5%</td>
</tr>
<tr>
<td>Lung – Large cell (neuroendocrine Ca)</td>
<td>8/36</td>
<td>22.2%</td>
</tr>
<tr>
<td>Lung – SCLC</td>
<td>8/15</td>
<td>53.3%</td>
</tr>
<tr>
<td>Malignant Mesothelioma</td>
<td>3/21</td>
<td>14.3%</td>
</tr>
<tr>
<td>Skin, normal</td>
<td>0/22</td>
<td>0%</td>
</tr>
<tr>
<td>Normal cutaneous adnexa (hair follicles and sebaceous glands)</td>
<td>0/8</td>
<td>0%</td>
</tr>
<tr>
<td>Skin – Squamous Ca</td>
<td>13/50</td>
<td>26.0%</td>
</tr>
<tr>
<td>Skin – Basal Cell</td>
<td>42/63</td>
<td>66.7%</td>
</tr>
<tr>
<td>Skin - Pilomatrixioma</td>
<td>10/44</td>
<td>22.7%</td>
</tr>
<tr>
<td>Skin – Merkel Cell Ca</td>
<td>4/6</td>
<td>66.7%</td>
</tr>
<tr>
<td>Skin - Benign Nevus</td>
<td>0/51</td>
<td>0%</td>
</tr>
<tr>
<td>Skin - Malignant Melanoma</td>
<td>11/34</td>
<td>32.4%</td>
</tr>
<tr>
<td>Skin - Dermatofibrosarcoma protuberans</td>
<td>0/5</td>
<td>0%</td>
</tr>
<tr>
<td>Skin - Granular Cell Tumor</td>
<td>0/8</td>
<td>0%</td>
</tr>
<tr>
<td>Urinary bladder, normal urothelial mucosa</td>
<td>0/20</td>
<td>0%</td>
</tr>
<tr>
<td>Urinary Bladder, Non-invasive</td>
<td>25/59</td>
<td>42.4%</td>
</tr>
</tbody>
</table>

Additional notes:
- Urinary Bladder, Non-invasive: 12 weak, 8 moderate, 5
- No correlation with
- Typical correlation with
- Strong correlation with
- Moderate correlation with
- Weak correlation with

Research notes:
- 18 from parotis, 8 submandibular, 8 sublingual, 8 small salivary glands
- 4 samples lost
- 2 weak, 1 moderate
- 3 weak, 2 moderate
- 9 weak, 4 moderate, 3 strong
- 16 weak, 6 moderate, 4 strong
- 2 weak, 1 moderate
- 3 weak, 3 moderate, 2 strong
- 3 weak, 2 moderate, 3 strong
- 2 weak, 1 moderate
- 8 weak, 5 moderate
- 35 weak (scattered cells +), 4 moderate, 3 strong moderate
- Only basaloid cells +
- 2 weak, 2 moderate
- 9 weak, 2 moderate
- 12 weak, 8 moderate, 5
- No correlation with

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MMSET expression in multiple tumors

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Count</th>
<th>Percentage</th>
<th>Staining Grade</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urothelial tumor (Ta)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary Bladder, Invasive</td>
<td>28/55</td>
<td>50.9%</td>
<td>6 weak, 16 moderate, 6 strong</td>
<td>The 6 tumors showing strong staining are all high-grade (G3)</td>
</tr>
<tr>
<td>Urothelial tumor (T2-4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urothelial Small CC</td>
<td>4/8</td>
<td>50.0%</td>
<td>All strongly +</td>
<td></td>
</tr>
<tr>
<td>Kidney, non-neoplastic</td>
<td>0/31</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal Oncocytoma</td>
<td>0/60</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal Papillary Ca</td>
<td>0/28</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal Chromophobe Ca</td>
<td>0/51</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal Conventional Clear Cell</td>
<td>0/64</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate, non-neoplastic</td>
<td>0/27</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate Ca</td>
<td>20/133</td>
<td>15.0%</td>
<td>All weak</td>
<td></td>
</tr>
<tr>
<td>Testis &amp; epididymis, non-neoplastic</td>
<td>0/19</td>
<td>0%</td>
<td></td>
<td>11 testis, 8 epididymis</td>
</tr>
<tr>
<td>Testis - Teratoma</td>
<td>14/53</td>
<td>26.4%</td>
<td>3 weak, 11 moderate</td>
<td>Mostly + in primitive, blastema-like tissue or neuroepthelium, no staining in more mature tissues</td>
</tr>
<tr>
<td>Testis - Seminoma</td>
<td>2/87</td>
<td>2.3%</td>
<td>Weak</td>
<td>Scattered reactive lymphocytes often positive</td>
</tr>
<tr>
<td>Testis - Non-seminomatous germinal cell tumor</td>
<td>0/44</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penis including corpus spongiosum, non-neoplastic</td>
<td>0/8</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penis Cancer (Squamous Ca)</td>
<td>5/44</td>
<td>11.4%</td>
<td>Weak</td>
<td></td>
</tr>
<tr>
<td>Ovary, normal stroma</td>
<td>0/12</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovary, corpus luteum</td>
<td>0/8</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovary, follicular cyst</td>
<td>0/8</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tuba uterina, normal mucosa</td>
<td>0/8</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovary – Serous Ca</td>
<td>25/59</td>
<td>42.4%</td>
<td>12 weak, 9 moderate, 4 strong</td>
<td>All but 1 nuclear grade 2-3 No correlation w/ nuclear grade No correlation w/ nuclear grade</td>
</tr>
<tr>
<td>Ovary – Mucinous Ca</td>
<td>4/42</td>
<td>9.5%</td>
<td>All weak</td>
<td></td>
</tr>
<tr>
<td>Ovary – Endometroid Ca</td>
<td>3/20</td>
<td>15.0%</td>
<td>2 moderate, 1 strong</td>
<td>No correlation w/ nuclear grade</td>
</tr>
<tr>
<td>Ovary – Brenner Tumor</td>
<td>0/38</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corpus Uteri, normal endometrium</td>
<td>3/33</td>
<td>9.1%</td>
<td>Weak (1 in proliferative phase, 2 in secretory phase)</td>
<td>10 in proliferative phase, 23 in secretory phase</td>
</tr>
<tr>
<td>Corpus Uteri – Endometroid Ca</td>
<td>22/54</td>
<td>40.7%</td>
<td>14 weak, 4 moderate, 4 strong</td>
<td>No Correlation w/ nuclear grade Not enough data to make correlation w/ nuclear grade</td>
</tr>
<tr>
<td>Corpus Uteri – Serous Ca</td>
<td>15/47</td>
<td>31.9%</td>
<td>5 weak, 6 moderate, 4 strong</td>
<td></td>
</tr>
<tr>
<td>Cervix Uteri</td>
<td>0/21</td>
<td>0%</td>
<td></td>
<td>10 samples from endocervix, 11 from ectocervix</td>
</tr>
<tr>
<td>Cervix Uteri – Squamous Ca</td>
<td>13/61</td>
<td>21.3%</td>
<td>6 weak, 2 moderate, 5 strong</td>
<td></td>
</tr>
<tr>
<td>Cervix Uteri –</td>
<td>14/44</td>
<td>31.8%</td>
<td>8 weak, 3 moderate, 3</td>
<td></td>
</tr>
</tbody>
</table>
## MMSET expression in multiple tumors

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Positive Cases</th>
<th>Percentage</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adenocarcinoma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervix Uteri – Adenosquamous Ca</td>
<td>0/3</td>
<td>0%</td>
<td>strong</td>
</tr>
<tr>
<td>Vagina, normal mucosa</td>
<td>0/5</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Vagina – Squamous Ca</td>
<td>4/19</td>
<td>21.1%</td>
<td>2 weak, 2 moderate</td>
</tr>
<tr>
<td>Vulva, normal mucosa</td>
<td>0/5</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Vulva – Squamous Ca</td>
<td>7/60</td>
<td>11.7%</td>
<td>2 weak, 5 moderate</td>
</tr>
<tr>
<td><strong>Breast, non-neoplastic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast – Ductal Ca</td>
<td>1/118</td>
<td>0.8%</td>
<td>Moderate</td>
</tr>
<tr>
<td>Breast – Lobular Ca</td>
<td>0/58</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Breast – Tubular Ca</td>
<td>0/56</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Breast – Mucinous Ca</td>
<td>5/57</td>
<td>8.8%</td>
<td>Weak 2 weak, 3 moderate</td>
</tr>
<tr>
<td>Breast – Medullary Ca</td>
<td>6/62</td>
<td>9.7%</td>
<td>3 weak, 3 moderate</td>
</tr>
<tr>
<td><strong>Lymph nodes, reactive non-neoplastic lymphatic tissue</strong></td>
<td>3/29</td>
<td>10.3%</td>
<td>moderate</td>
</tr>
<tr>
<td><strong>Spleen, non-neoplastic</strong></td>
<td>0/8</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td><strong>Tonsil, reactive non-neoplastic lymphatic tissue</strong></td>
<td>11/15</td>
<td>73.3%</td>
<td>weak</td>
</tr>
<tr>
<td><strong>Hodgkin’s Lymphoma (HL)</strong></td>
<td>0/36</td>
<td>0%</td>
<td>7 weak (scattered + cells), 1 strong (diffusely +)</td>
</tr>
<tr>
<td>Non-Hodgkin Lymphomas (NHL)</td>
<td>8/9</td>
<td>88.9%</td>
<td>High-grade diffuse large B cell lymphomas (DLBCL)</td>
</tr>
<tr>
<td><strong>Thymus, non-neoplastic</strong></td>
<td>8/8</td>
<td>100%</td>
<td>3 weak, 3 moderate, 2 strong</td>
</tr>
<tr>
<td>Thymoma</td>
<td>0/51</td>
<td>0%</td>
<td>Reactive lymphocytes often +, but not epithelial tumor cells</td>
</tr>
<tr>
<td><strong>CNS: Cerebellum, non-neoplastic</strong></td>
<td>0/16</td>
<td>0%</td>
<td>8 grey &amp; 8 white matter</td>
</tr>
<tr>
<td>CNS: Cerebrum, non-neoplastic</td>
<td>0/16</td>
<td>0%</td>
<td>8 grey &amp; 8 white matter</td>
</tr>
<tr>
<td><strong>CNS - Astrocytoma</strong></td>
<td>0/44</td>
<td>0%</td>
<td>From low- to high-grade (glioblastoma)</td>
</tr>
<tr>
<td><strong>CNS - Ependymoma</strong></td>
<td>0/9</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>CNS (cerebellum-) Medulloblastoma</td>
<td>0/4</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td><strong>CNS - Oligodendroglioma</strong></td>
<td>4/26</td>
<td>15.4%</td>
<td>Weak</td>
</tr>
<tr>
<td><strong>Aorta, non-neoplastic</strong></td>
<td>0/16</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td><strong>Vessels in soft tissues (samples w/ vascularised striated or smooth muscle tissue)</strong></td>
<td>0/114</td>
<td>0%</td>
<td></td>
</tr>
</tbody>
</table>
**MMSET expression in multiple tumors**

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiosarcoma</td>
<td>0/4</td>
<td>0%</td>
</tr>
<tr>
<td>Normal striated muscle</td>
<td>0/29</td>
<td>0%</td>
</tr>
<tr>
<td>Normal Myocardium</td>
<td>0/21</td>
<td>0%</td>
</tr>
<tr>
<td>Normal smooth muscle</td>
<td>0/72</td>
<td>0%</td>
</tr>
<tr>
<td>Leiomyoma</td>
<td>0/25</td>
<td>0%</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>3/24</td>
<td>12.5%</td>
</tr>
<tr>
<td>Desmoid tumor</td>
<td>0/6</td>
<td>0%</td>
</tr>
<tr>
<td>GIST</td>
<td>0/35</td>
<td>0%</td>
</tr>
<tr>
<td>Hemangiopericytoma</td>
<td>0/6</td>
<td>0%</td>
</tr>
<tr>
<td>Chondrosarcoma</td>
<td>0/3</td>
<td>0%</td>
</tr>
<tr>
<td>Neurofibroma</td>
<td>0/56</td>
<td>0%</td>
</tr>
<tr>
<td>Malignant Schwannoma</td>
<td>0/13</td>
<td>0%</td>
</tr>
<tr>
<td>Malignant Fibrous</td>
<td>8/24</td>
<td>33.3%</td>
</tr>
<tr>
<td>Histiocytoma (MFH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal fat tissue</td>
<td>0/8</td>
<td>0%</td>
</tr>
<tr>
<td>Liposarcoma (LPS)</td>
<td>4/12</td>
<td>33.3%</td>
</tr>
</tbody>
</table>

Intensity of MMSET nuclear immunostaining as described in Materials and Methods. Ca: carcinoma.
Figure 1

A

9A6

1-119 a.a.

MMSET type I, 647 a.a.

MMSET type II, 1365 a.a.

B

Control

MMSET-I shRNA

250

98

64

MMSET II

MMSET I

b-TUBULIN

C

Control

MMSET I+II shRNA

Pre-SET

Post-SET

Cdk-H

NLS

RE-IIBP, 584 a.a.

PWPP

HMG

additions
Figure 2

Plasma cells

MM n=39
100%
75%
50%
25%
0%
P=5.3x10^-4

NPC n=33

MMSET score 0

Multiple myeloma 31
MMSET 3

Multiple myeloma 39
MMSET score 3

Research.
Figure 3

A

Lung

B

Stomach

C

Anal canal

MMSET

$\square$ 3

$\square$ 2

$\square$ 1

$\square$ 0

$\text{N bronchus n=16}$

$\text{Sq Ca n=67}$

$\text{LCNEC n=36}$

$\text{SCLC n=15}$

$\text{BAC n=11}$

$\text{A Ca n=94}$

$\text{N parenchyma/pleura n=40}$

$\text{Malignant mesothelioma n=21}$

$\text{N bronchus n=16}$

$\text{Intestinal ACa n=54}$

$\text{Diffuse ACa n=47}$

$\text{Intestinal ACa n=54}$

$\text{Diffuse ACa n=47}$

$\text{N mucosa n=21}$

$\text{N mucosa n=21}$

$\text{Normal gastric mucosa}$

$\text{Normal anal mucosa}$

$\text{Normal anal mucosa}$

$\text{Squamous carcinoma}$

$\text{Adenocarcinoma}$

$\text{LCNEC}$

$\text{SCLC}$

$\text{Intestinal adenocarcinoma}$

$\text{Anal basaloid squamous carcinoma}$

$p=8.2\times10^{-4}$

$p=9.8\times10^{-4}$

$p=1.0\times10^{-4}$

$p=0.037$

$p=1.0\times10^{-4}$

$p=0.010$
**Figure 4**

**A** Colon, cohort 1

![Graph showing distribution of normal mucosa, dysplasia, low-grade, dysplasia, high-grade, and adenocarcinoma](image)

- Normal mucosa, n=39
- Dysplasia, low-grade, n=51
- Dysplasia, high-grade, n=37
- Adenocarcinoma, n=114

**B** Colon, cohort 2

![Graph showing distribution of normal mucosa and adenocarcinoma](image)

- Normal mucosa, n=13
- Adenocarcinoma, n=1206

**C** Stage

- Stage pT1, n=48
- Stage pT2, n=176
- Stage pT3, n=739
- Stage pT4, n=190

**D** Survival

![Graph showing survival probability over time](image)

- MMSET 3
- MMSET 2
- MMSET 1
- MMSET 0

**D** Invasive margin

- Pushing, n=416
- Infiltrating, n=734

**Legend:**
- pT1: 100%
- pT2: 75%
- pT3: 50%
- pT4: 25%
- pN0: 0%
- pN1: 25%
- pN2: 50%
- pN3: 75%
- pN4: 100%

**Statistical Significance:**
- p = 5.5 x 10^{-8}
- p = 1.7 x 10^{-18}
- p = 4.8 x 10^{-13}
- p = 2.1 x 10^{-3}
- p = 5.5 x 10^{-8}
- p = 1.7 x 10^{-18}
- p = 4.8 x 10^{-13}
- p = 0.023
- p = 1.5 x 10^{-4}
- p = 7.0 x 10^{-3}
- p = 3.3 x 10^{-5}

**Log-rank Test:**
- p = 7.4 x 10^{-6}
Figure 5

A. Urinary bladder, cohort 1

B. Urinary bladder, cohort 3

C. Grade

D. Progression

1. MMSET
2. Normal urothelial mucosa
3. Urothelial tumor, low-grade
4. Urothelial tumor, high-grade
5. Small cell carcinoma

Cohort 3, pool, n=1293

- 0-40 n=23
- 41-50 n=65
- 51-60 n=237
- 61-70 n=469
- 71-80 n=383
- >80 n=173

- p=3.8x10^-3
- p=4.6x10^-3
- p=3.4x10^-3
- p=1.3x10^-5
- p=1.0x10^-6
- p=1.0x10^-6
- p=7.8x10^-3

- log-rank test, p=6.3x10^-4

- pTa n=50
- pT1 n=30
- pT2-pT4 n=428

- G1 n=14
- G2 n=56
- G3 n=58

- Solid n=32
- Papillary n=93
The histone methyltransferase and putative oncoprotein MMSET is overexpressed in a large variety of human tumors


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