

LETTER TO THE EDITOR

MMSET is the key molecular target in t(4;14) myeloma

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The t(4;14)(p16.3;q32.3) is found in 15% of presenting multiple myeloma (MM) cases and is associated with a significantly worse prognosis than other biological subgroups. As a consequence of the translocation, two genes are aberrantly expressed, the fibroblast growth factor receptor 3 (*FGFR3*) and a multiple myeloma SET domain containing protein, *MMSET* (*WHSC1/NSD2*), both of which have potential oncogenic activity.¹ Importantly, *FGFR3* shows only weak transforming activity and is eventually lost in 30% of patients,² suggesting that it is not the main oncogenic factor. In contrast, *MMSET* gene overexpression is universal, and when it is knocked down experimentally, there is inhibition of proliferation, induction of apoptosis and alteration of cell adhesion,^{3–5} suggesting it is central to the pathogenesis of this subtype of MM. *MMSET* is known to have histone methyl transferase activity⁶ and is deregulated early on in the genesis of developing myeloma, and could therefore constitute a good therapeutic target. The *MMSET* locus in t(4;14) myeloma patients has a complicated genomic structure and after translocation events and RNA splicing, a number of different transcripts are generated (Figure 1). This genetic complexity of *MMSET* has been added to recently by the discovery of the H/ACA box RNA *ACA11* (*SCARNA22*), that has been found within intron 20 of *MMSET* and is also overexpressed in the t(4;14) subgroup.⁷ This small RNA has been suggested to be key to the pathogenesis of t(4;14) MM, raising the question that it may constitute the main therapeutic target.

The box H/ACA RNAs are a group of small nucleolar RNA (snoRNA) conserved from Archea to mammals. These RNAs are generally associated with a multi-protein complex, and usually function as a guide to the site-specific pseudouridylation of rRNA and spliceosomal small nuclear RNAs. However, they are also involved in other regulatory complexes, such as telomerase,⁸ but their full biological roles have not been completely elucidated. More than 90% of human snoRNA genes are encoded within spliced introns,⁹ and their expression is closely linked to the transcription of the host gene.^{9,10} After host gene transcription, the intronic snoRNAs are trimmed to a mature form by exonucleolytic activities. The mRNA splicing machinery may chaperone snoRNA post-transcriptional maturation steps, but in some cases, these steps are splicing-independent.¹¹ However, there are a few cases in mammals where snoRNAs are independently transcribed, for instance, the gene for the telomerase RNA component (*terC*) or the RNAs involved in the pre-rRNA endonucleolytic processing,^{8,9} but this is a rare event.

In a series of experiments it has been shown that *ACA11* knockdown impairs cell proliferation and deregulates the oxidative stress response, and its overexpression downregulates the transcription of ribosomal protein genes.⁷ The same group, on the basis of experiments showing that t(4;14) cell lines knocked out for *MMSET*, either on the translocated allele (TKO) or on the non-translocated allele (NTKO), have lower *ACA11* levels compared with their parental cell line (KMS11) with a normal overexpressed *MMSET*, suggested that *ACA11*, rather than *MMSET*, is the key pathogenic gene in t(4;14) MM. In previous studies, the same TKO model system had been used to prove the oncogenic effect of

MMSET in myeloma,^{5,6} however, in these more recent experiments where *ACA11* was knocked down, the key implication of the work seemed to shift the pathogenic importance from *MMSET* to *ACA11*.⁷

However, when we examined these results in detail, some of the results do not seem to accurately reflect what is understood about the TKO cell line system biology. In particular, the *MMSET* gene was erroneously reported to be deleted, whereas, in fact, the TKO and NTKO cell lines were generated from the parental KMS11 t(4;14) line, by deleting *MMSET* exon 7 only⁵ (Figure 1). This deletion creates a frame shift that introduces an earlier STOP codon, leaving the level of *MMSET* mRNA unaltered, and in theory, *ACA11* untouched. Moreover, both TKO and NTKO cell lines express the *MMSET* isoform REIIBP.⁵ *REIIBP* mRNA is transcribed from an intronic promoter downstream of both the TKO and NTKO knockout mutation¹² (Figure 1), and harbors intron 20, the location of the *ACA11* gene. In order to reconcile the observations reported in the paper with what we understand about the biology of *MMSET* and other human intronic snoRNAs, we would have to hypothesize that *ACA11* biogenesis in myeloma is different. It could be possible that *ACA11*, even though it is localized within an *MMSET* intron, could have an independent transcription start site distinct from the *MMSET/REIIBP* gene, or alternatively it may be regulated at the post-transcriptional level. However, as TKO, NTKO and KMS11 are con-isogenic lines, differing only for the level of functional *MMSET*, differential *ACA11* regulation could only be due to a direct or indirect role of *MMSET*.

In order to resolve these discrepancies, we investigated *MMSET* and *ACA11* expression in 153 myeloma patients as well as in the same set of con-isogenic cell lines. Statistical analysis showed that there is a good correlation between the expression of both genes (Figure 2a), suggesting that their regulation is interdependent. A similar result was found by an independent study recently.¹³ This observation was also corroborated by ENCODE RNA sequencing (RNA-seq) data,¹⁴ in which *MMSET* and *ACA11* RNAs expression in non-myeloma lines were also frequently co-expressed. The results of this work are consistent with *ACA11* biogenesis being via a classical intronic mechanism. Further, we went on to show that *ACA11* expression was indeed lower in TKO cells, using RNA-seq on TKO and its parental KMS11 cell lines. We found that both lines had equal levels of signal coming from *MMSET* exons, (Figure 2b). As an internal control, we were able to demonstrate that exon 7 signal levels were reduced in TKO. We describe an intronic signal peak present in both cell lines, derived from intron 20 (Figure 2b), which is of comparable intensity and perfectly overlapping with the position of the *ACA11* gene. This result demonstrates that in both lines the snoRNA is intact and is present in equal amount.

To confirm and better characterize this result, we designed and used a qRT-PCR test and applied it to total RNA from KMS11, TKO and NTKO looking at the expression of *ACA11* and *MMSET*. In this experiment, we compared these values to those from HeLa, RPMI-8226 (t(4;14) negative MM line) and TKO::MMSET (TKO line virally transduced with the intronless cDNA for *MMSET*, and hence not carrying *ACA11*), (Figure 2c). We demonstrated a marked difference in *MMSET* and *ACA11* expression between t(4;14) positive and negative lines. However, in contrast, we could not demonstrate a difference in *MMSET* mRNA in TKO, KMS11 and NTKO lines, even if these lines have different level of *MMSET* protein.^{5,6}

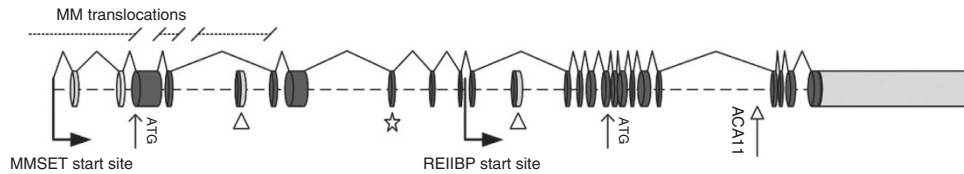


Figure 1. MMSET genetic map on chromosome 4. The position of the different translocation events in MM is shown by slashed lines. Gray and black ovals indicate non-coding and coding exons, respectively; dotted straight line between exons represent the introns. Splicing event for the canonical longest *MMSET* isoform is shown by joining lines between exons. Exons 4a and 11, shown by white triangles, are used only for alternative splicing and are not present in the longest *MMSET* isoform. Exon 7, deleted in TKO and NTKO lines, is indicated by a white star. Transcription start sites and first translated codons (ATG) for *MMSET* and *REIIBP* are indicated, respectively, by bent black arrows and open arrows. Genomic position for *ACA11* is shown by a white arrow.

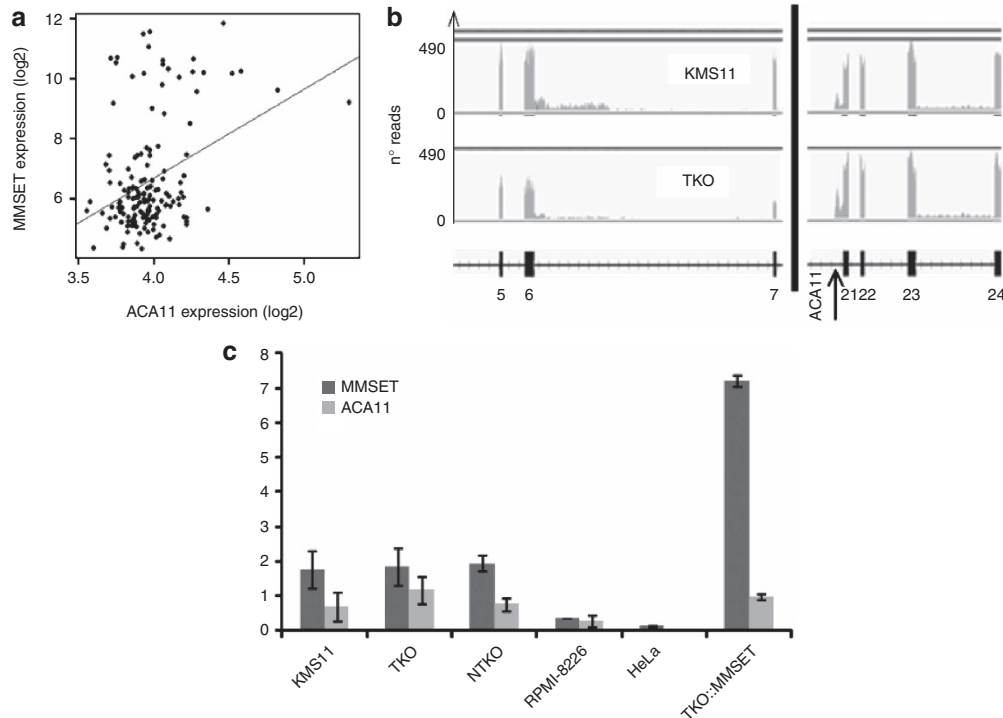


Figure 2. Correlation between *MMSET* expression and *ACA11* expression. **(a)** Scatter plot showing a positive correlation between *ACA11* expression (by Affymetrix GeneChip microRNA arrays v1.0; Affymetrix, Santa Clara, CA, USA) and *MMSET* (222777_s_at) expression (by Affymetrix HG-U133 Plus 2.0 arrays) in 153 subjects from Myeloma IX with the Pearson's correlation coefficient (r) of 0.36 and the two-tail P -value of 0.000005 **(b)** *ACA11* is expressed in *MMSET* knockout cell lines. RNA-seq analysis was performed using the methods and tools described here¹⁵ and visualized using IGV (Integrative Genome Viewer, Broad Institute). Number below the filled boxes show the relative position for *MMSET* exons; straight lines between exons represent the introns. Arrow head indicates genomic *ACA11* position. KMS11 line was provided by Dr Otsuki (Kawasaki Medical School, Japan), TKO and NTKO by J Lauring (Johns Hopkins, Baltimore). **(c)** qRT-PCR values for *MMSET* and *ACA11*. The results are expressed relative to human β -Actin. Contamination for genomic DNA was tested by performing cDNA synthesis in the absence of reverse transcriptase and amplifying the samples with primers for a control region on chromosome 12. The error bars depicted are the standard deviations from two to four separate RNA extractions and cDNA syntheses. TKO::MMSET was generated by viral transduction cloning *MMSET* cDNA into pRRLSIN lentivector.

Taking the RNA-seq and qRT-PCR data as whole, they show that *ACA11* expression directly mirrors *MMSET* expression level, highlighting the correlation demonstrated in primary patient material (Figure 2a). We also show that *ACA11* expression is neither deregulated in the TKO nor in the NTKO cell line compared with parental KMS11. This result is compatible with the *ACA11* snoRNA being generated by excision from the *MMSET* mRNA. Alternatively, if *ACA11* had an independent promoter from *MMSET* isoforms, it must be under the control of the same enhancer that regulates *MMSET* and *REIIBP* expression (the *IgH* enhancer or the *MMSET* enhancer in non-t(4;14) samples). Interestingly, we saw that in TKO::MMSET, the *ACA11* level was very close to those of TKO and KMS11, even if *MMSET* levels were virally transduced to be

four-times higher. This observation shows that *MMSET* has no role in directly regulating *ACA11* gene expression or in regulating its post-transcriptional maturation steps.

Overall, the data presented here are consistent with *MMSET* being the key pathologic mediator in t(4;14) myeloma.⁶ We do, however, think that *ACA11* has an important role in t(4;14) pathogenesis, because evolutionary pressure seems to have kept *ACA11* inside the *MMSET* locus, and often, intronic RNA are found to be involved in the same biochemical pathway as their host gene.⁹

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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