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SET alpha and SET beta mRNA isoforms in chronic lymphocytic leukaemia

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Summary

Alteration in RNA splicing is implicated in carcinogenesis and progression. Mutations in spliceosome genes and alternative splicing of other genes have been noted in chronic lymphocytic leukaemia (CLL), a common B cell malignancy with heterogeneous outcomes. We previously demonstrated that differences in the amount of SET oncoprotein (a physiological inhibitor of the serine/threonine phosphatase, PP2A) is associated with clinical aggressiveness in patients with CLL. It is unknown if alternative splicing of gene transcripts regulating kinases and phosphatases affects disease pathobiology and CLL progression. We show here for the first time that mRNA levels of the alternatively spliced SET isoforms, *SETA* and *SETB* (SETα and SETβ), significantly correlate with disease severity (overall survival and time-to-first-treatment) in CLL patients. In addition, we demonstrate that relative increase of *SETA* to *SETB* mRNA can discriminate patients with a more aggressive disease course within the otherwise favourable CLL risk classifications of *IGHV* mutated and favourable hierarchical fluorescence *in situ* hybridisation groups. We validate our finding by showing comparable relationships of *SET* mRNA with disease outcomes using samples from an independent CLL cohort from a separate institution. These findings indicate that alternative splicing of *SET*, and potentially other signalling cascade molecules, influences CLL biology and patient outcomes.

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Author contributions

Planned the work: DMB, DJC, LZR, TJK, DRF, JBW; recruited patients: DMB, TJK, CD, DRF, JBW; performed laboratory analyses: LZR, EG, YC, ADV, CD; performed statistical analyses: DZ, KO, XW; wrote/edited the manuscript: DMB, DJD, DJC, LZR, TJK, DZ, KO, XW, DRF, JBW.

Disclosures. DJC and JBW have a patent regarding use of SET as a predictor of CLL and NHL outcomes. DMB has no conflicts in regarding to work under consideration for publication. For work outside of the publication, DMB has been a consultant for Abbvie, Genentech, Pharmacyclics, Teva, TG Therapeutics; and received clinical trial research support from Abbvie, TG Therapeutics, Gilead, DTRM, and Beigene.

Keywords

Chronic lymphocytic leukaemia; SET; PP2A; alternative RNA splicing; phosphatase

Introduction

Chronic lymphocytic leukaemia (CLL) is characterized by marked clinical heterogeneity whereby some patients never require treatment and have life expectancies similar to their age-matched peers, while other patients experience rapid progression and treatment resistance with significantly shortened survival.(The International CLL-IPI Working Group 2016, Chiorazzi*, et al* 2005, Parikh*, et al* 2016, Pflug*, et al* 2014, Thompson*, et al* 2016) Despite significant advances in CLL, including available novel therapeutics, there remains great variability in outcomes; a better understanding of disease pathobiology is needed to identify these disparate groups.

Alternative RNA splicing can alter normal cellular functions, cooperating with other oncogenic progresses to drive leukaemogenesis or cancer progression.(Hanahan and Weinberg 2011, Surget*, et al* 2013) In both adult and paediatric AML, for instance, relative change in the expression of the different alternatively spliced isoforms of *BIRC5* (*survivin),* an inhibitor of apoptosis protein, is related to risk of aggressive disease relapse and rapid progression with shortened patient survivals.(Beghini*, et al* 2000, Moore*, et al* 2014, Wagner*, et al* 2006) In lymphoid malignancies, such as CLL, mutations in certain spliceosome-related genes and alternative splicing also appear to modulate disease severity. For example, in CLL, mutations in the 3' region of *NOTCH1* induce aberrant *NOTCH1* mRNA splice variants(Puente*, et al* 2015) and *SF3B1* mutations induce alternative splicing in numerous genes.(Wang*, et al* 2017) Both *NOTCH1* and *SF3B1* mutations are associated with inferior CLL-specific clinical outcomes.

Recent advances in the understanding of the critical role that B-cell receptor signalling and modulation of apoptosis play in CLL has led to development and use of novel therapies in this malignancy.(Byrd*, et al* 2013, Furman*, et al* 2014) SET protein, a potent endogenous inhibitor of protein phosphatase 2A (PP2A), is a known regulator of oncogenic signalling, apoptosis and the cell cycle. For this reason, we previously evaluated SET protein in CLL, and demonstrated that the SETα and SETβ proteins are expressed at higher levels in malignant CLL and non-Hodgkin lymphoma (NHL) cells compared to normal B cells. (Christensen*, et al* 2011)

Because of the growing appreciation of RNA splicing as an oncogenic mechanism, we here measured *SETA* and *SETB* mRNA expression in CLL cells from patients attending the Duke University or Durham Veteran's Affairs Medical Centers (Duke/DurVAMC), and from patients in an independent cohort of CLL patients from the Moores Cancer Center of the University of California San Diego (UCSD). Additionally, we evaluated the extent to which relative expression of SET isoforms influences disease progression within the context of established clinical and molecular prognostic factors. This evaluation connects the oncogenic processes of alternative splicing and second messenger signalling on the cellular

level with the heterogeneity of disease aggressiveness that can be observed in patients with CLL.

Methods

Patient Inclusion & CLL cell preparation

Patients diagnosed with CLL at Duke/DurVAMC and the UCSD, were enrolled on Institutional Review Board-approved protocols to collect clinical data and peripheral blood. All patients provided written, informed consent prior to enrolment in accordance with the Declaration of Helsinki. Indications for treatment were based on the International Workshop on CLL guidelines(Hallek, *et al* 2008), but selection and timing of therapy was at the discretion of the treating physicians. Some patients had treatment prior to being evaluated at the participating centres. Time to first treatment (TTFT) was defined as the length of time from the date of CLL diagnosis to initiation of first treatment. Overall survival (OS) was defined as the length of time from diagnosis to death from any cause. The follow-up time was the time from initial diagnosis until the time of death or the time of last contact.

CLL cells from the first collected sample were purified as previously published. In brief, Duke/DurVAMC CLL cells were enriched using the RosetteSep® B cell enrichment cocktail (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's directions, yielding a CLL purity by flow cytometry ($CD5^+CD19^+$ B-cells) of greater than 95%(Christensen, *et al* 2011). At UCSD, the leukaemia cells were isolated by ficoll/Hypaque density gradient technique.(Rassenti, *et al* 2004) Patient characteristics and prognostic markers were obtained from clinical records, with the *IGHV* mutation status and CD38 expression levels determined on the Duke/DurVAMC samples as previously described.(Volkheimer, *et al* 2007) In every case, the first sample available was utilized to calculate CD38 expression levels and determine positivity (≥30% considered positive).

Quantitative polymerase chain reaction measurement of SET *mRNA*

Primers and probes for quantitative real time polymerase chain reaction (RT-qPCR) designed to amplify only the alpha or beta forms of *SET* are described in Figure 1 and its legend. Total RNA was extracted from pellets of 10×10^6 CLL cells using RNeasy Mini Kit (Qiagen, Germantown, MD) following the manufacturer's instructions. cDNA was prepared with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using the manufacturer's protocol, with inclusion of the QiaShredder (Qiagen) homogenization and the on-column DNase digestion with RNase-Free DNase Set (Qiagen). After isolation, the nucleic acid concentration of each sample was determined using a NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA). Quantitative PCR was performed with 1 μg of RNA reverse transcribed using Transcriptor Universal cDNA Master (Roche, Indianapolis, IN) followed by utilization of TaqMan master mix (Applied Biosystems) and following the manufacturer's protocol. Fold changes were calculated using ΔΔCt method. For quantification, *SETA* was cloned onto pCMV6-XL5 plasmid and *SETB* was cloned in the pcDNA3.1 plasmid. Absolute copy numbers were calculated based on OD and number of bases. Serial ten-fold dilutions of plasmid DNA were used in the qPCR to produce a standard curve for absolute quantification of CLL samples.

Statistical Analyses

For the primary inferential analysis of TTFT and OS, the *SETA/SETB* mRNA ratio was analysed as a continuous variable. The dichotomized data were used for visual display of the effects in Kaplan-Meier plots (high vs. low *SETA/SETB* mRNA ratio); an individual patient's CLL cell *SETA/SETB* mRNA ratio at or above the median ratio for the group was considered a "high *SET* ratio;" if the *SETA/SETB* was below the median ratio it was considered a "low *SET* ratio." The secondary analysis was to test for an interaction effect of *SETA/SETB* ratio with other established CLL risk biomarkers.

The primary inferential analyses were based on the continuous variables, and the dichotomized data were used for visual display of the effects in Kaplan-Meier plots. The secondary analysis was to test for an interaction effect of *SETA/SETB* mRNAratio with other established CLL risk biomarkers including Rai stage, *IGHV* mutation status, CD38 positivity (≥30% considered positive) and favourable *vs.* unfavourable A(unfavourable = del(17p) or del(11q) alone or in any combination, or three or more abnormalities) with respect to TTFT and OS. This interaction testing was to determine if the effect of the effect of the *SETA/SETB* mRNA on patient outcomes was dependent on the other prognostic variables (positive interaction).

The distributions of continuous variables were assessed using boxplot, and log2 transformation was used for skewed variables. All statistical analyses were conducted using the R statistical environment v3.3.2 (https://www.r-project.org/). Cox proportional hazard regression was used for the time to event analyses using the R extension package survival v2.40. (Cox & Oakes 1984, Therneau 2016) Score test p-values and Wald test p-values were reported for univariate analysis and interaction analyses, respectively. P-values were not adjusted for multiple testing.

Results

Patient characteristics

The Duke/DurVAMC combined population was analysed independently from the validation cohort of UCSD patients. The median *SETA/SETB* mRNA ratio (*SETA/B*) of the Duke/DurVAMC population was 0.94 (range = 0.13 – 33.50) and the median for the UCSD population was 0.93 (range = 0.24 – 19.20) (p = not significant). Patient characteristics of the analysed Duke/DurVAMC (n=307) and UCSD cohorts (n=166) are summarized in Table I. FISH is reported hierarchically as detailed in Table I footnotes. The cohorts were representative of other published CLL populations with regards to age and distribution of prognostic factors.(Molica, *et al* 2016) There were no significant differences between the Duke/DurVAMC and UCSD patients relative to sex (32% vs. 40% female, respectively, p=0.1) and low risk Rai stage 0 at diagnosis. Patients in the San Diego cohort had higher CD38 positivity than those in the Duke/DurVAMC cohort (28% vs. 19%; p=. 047), but the percent of patients with the adverse marker unmutated *IGHV* (UM-*IGHV*) was not significantly different between the groups (43% *vs.* 39%, p=0.529).

Increased SETA isoform mRNA relative to SETB isoform mRNA is associated with CLL aggressiveness

The median follow-up time for the Duke/DurVAMC patients was 16.3 years. Established biomarkers and the *SETA/B* mRNA ratio were tested in univariate analyses for TFTT in both cohorts. As others and we have noted before, UM-*IGHV*, CD38 positivity (CD38+) and Rai stage I-IV at diagnosis were significantly associated with shorter times to first treatment in both groups.(Chiorazzi*, et al* 2005, Weinberg*, et al* 2007) In the Duke/DurVAMC cohort, patients with unfavourable FISH [del(17p), del(11q) or three or more than 3 cytogenetic abnormalities by FISH] had a significantly shorter TTFT and OS (Table II), but those with del(17p) alone did not have a shorter TTFT. The number of patients with del(17p) was small, and this may have limited the power of this analysis.

We measured *SETA* and *SETB* mRNA using isoform-specific RT-PCR, and evaluated both isoforms with respect to their influence on clinical outcomes by calculating a ratio of *SETA* to *SETB.* Patients with higher *SETA/B* mRNA ratio assessed as a continuous variable had significantly shorter TTFT, [hazard ratio (HR) 1.2, p=0.001]. Results of analyses of the *SETA/B* mRNA ratio as a dichotomous variable in TTFT and OS (for illustrative purposes only) is displayed as Kaplan-Meier plots in Figure 2 and included in Table II. Patients with a dichotomized high *SETA/B* mRNA ratio had a median TTFT of 4.3 years (3.5–5.5), while patients with a low *SETA/B* mRNA ratio had a median TTFT of 9.3 years (5.9–14.0); [HR 1.7 (1.3–2.3), p<0.001]. Patients from the Duke/DurVAMC cohort with dichotomized high *SETA/B* mRNA ratio had a significantly shorter OS [15.3 years (11.9–18.1)] compared to patients with low *SETA/B* mRNA ratio [22.6 years (19.3-)], [HR 1.7 (1.2–2.5), p=0.005]. To help validate our *SET* mRNA PCR assay, we assessed 276 individual purified CLL cell samples (Christensen*, et al* 2011) for both SETA/B protein by immunoblot and *SETA/B* mRNA by RT-PCR. Results showed significant correlation of the SETA/B protein by immunoblot assay and *SETA/B* mRNA by RT-PCR assay ($p < 0.0005$, $R = 0.206$).

As some CLL biomarkers can evolve and be enriched post-chemoimmunotherapeutic treatment, we additionally analysed the patients from the Duke cohort who had a sample for *SET* mRNA PCR analysis collected prior to any therapeutic intervention. In all cases, we utilized the first sample that was available. In the Duke cohort, 233 patients had TTFT (or follow-up with no treatment) information available and had a CLL sample for qPCR analysis of *SET* mRNA isoforms collected prior to the first treatment. Of these 233 patients, 116 eventually were treated. As a continuous variable, the HR for TTFT for a higher *SET* mRNA ratio in this group was 1.05 (95% CI (1.01–1.09)), p=0.01. Similarly, 234 patients in the Duke cohort had OS data available and had their CLL sample for analysis drawn before any therapy was given. Sixty-two of these patients later died. The HR for a higher *SET* mRNA ratio and death in these patients was 1.06 (1.02–1.11), p=0.004). Therefore *SET* mRNA isoform ratio retains its prognostic significance for TTFT and OS even when tested in patients of the Duke cohort who had their samples taken before any chemoimmunotherapy was given.

High SETA *isoform mRNA relative to* SETB *isoform mRNA association with shortened TTFT is confirmed in a separate, independent CLL cohort*

Analysis of *SET* mRNA in cells of CLL patients from UCSD revealed that a high *SETA/B* mRNA ratio as a continuous variable was also significantly associated with shorter TTFT [HR 1.3 (1.0–1.6), p=0.02]. Kaplan-Meier plots of the UCSD cohort analysed with *SETA/B* mRNA ratio as a dichotomous variable for illustration are included in Figure 2, with Table III listing TTFT and OS for the high and low *SETA/B* mRNA ratio groups. The median TTFT was 6.1 (4.8–8.1) years in the dichotomized high *SETA/B* mRNA ratio group *vs.* 10.3 (8.4–15.6) years in those with a low *SETA/B* mRNA ratio [HR 1.8 (1.2–2.7), p=0.006]. There was no significant association of high *SETA/B* mRNA ratio as a continuous or dichotomous variable with shortened OS in the UCSD patients. The lower number of deaths in the UCSD cohort may have contributed to the difference in effect on OS seen between the two cohorts.

Increased SETA *isoform mRNA relative to* SETB *isoform mRNA provides risk stratification in favourable CLL prognostic groups*

Clinically established prognostic markers, such as Rai stage, CD38 positivity, *IGHV* mutation status and FISH profile, do not fully predict CLL patient outcomes. This indicates that additional modifiers of disease aggressiveness probably exist. We therefore first tested for interactions of the dichotomous *SETA/B* mRNA ratio with *IGHV* mutation status, FISH group, CD38 positivity and Rai staging in the Duke/DurVAMC cohort. As expected, CLL patients with UM-*IGHV* have a shorter TTFT compared to patients with mutated *IGHV* (M-*IGHV*) [3.1 vs. 11.9 years, HR=3.34, p<0.001; Figure 3]. Patients with M-*IGHV* and a high *SETA/B* mRNA ratio had a shorter TTFT compared to those with M-*IGHV* and a low *SETA/B* mRNA ratio. However, *SETA/B* mRNA ratio did not risk stratify the unfavourable risk UM-*IGHV* group of patients ($p_{interaction}$ = 0.01).

As with the UM-*IGHV* patients, CLL patients with unfavourable FISH had a shorter TTFT compared to those with favourable FISH [4.6 vs. 7.3 years, HR=1.6, p<0.001]. In the favourable risk FISH group, a high *SETA/B* mRNA ratio predicted a shorter TTFT compared to low *SETA/B* mRNA ratio, but this risk stratification was not seen in the unfavourable FISH group ($p_{interaction}$ = 0.01). *SETA/B* mRNA ratio appeared to risk-stratify CLL patients when combined with other established prognostic markers in the Duke/DurVAMC cohort (Rai stage and CD38 – Figure 4) and the UCSD cohort (*IGHV* mutation status or the FISH group – Figure 5), although there were no significant interactions in these analyses. Interaction of *SET* isoform mRNA ratio with CD38 may part be partly affected by the known variability of CD38 in patients across time, while *IGHV* and FISH have more consistently been shown to be independent prognostic markers, including in a validated international prognostic index, the CLL-IPI. (The International CLL-IPI Working Group, 2016)

The positive interactions of *IGHV* and FISH with *SET* mRNA isoforms indicates that a high *SETA/B* mRNA ratio is associated with inferior clinical outcomes and can be combined with established prognostic markers for more refined estimation of patient prognosis. The interaction between *SETA/B* mRNA ratio and *IGHV* mutation status or FISH group suggests

that SET may cooperate with other proteins or cellular pathways in certain biologically-defined patient subgroups, thereby influencing disease aggressiveness primarily for those patients.

Discussion

We present here that expression of the alternatively spliced *SET* mRNA isoforms is associated with clinical outcomes in patients with CLL. We show for the first time that a relative increase in the expression of *SETA* mRNA isoform (high *SETA/B* mRNA ratio) is associated with shortened TTFT and OS in CLL patients. Using an independent group of CLL patients from a separate institution, we also verified that patients with high *SETA* relative to *SETB* isoform mRNA expression have inferior outcomes with faster progression to treatment indications (Figure 2). We believe this provides novel insights into CLL disease biology, potentially through SET's role in dysregulation of the critical kinase-phosphatase balance. However, the dominant function(s) of each SET isoform and the comparative potency between isoforms for these various SET functions in malignant cells is not defined. Alternative splicing of *SET* mRNA isoforms may provide an additional layer of oncogenic dysregulation in CLL.

SET overexpression and involvement in tumourigenesis has been described in many cancers, including lung, prostate, ovarian and head and neck cancers, and leukaemias(Agarwal, *et al* 2014, Carlson, *et al* 1998, Christensen, *et al* 2011, Cristobal, *et al* 2012, Cristobal, *et al* 2015, Cristobal, *et al* 2017, Gonzalez-Alonso, *et al* 2015, Liu, *et al* 2015, Neviani, *et al* 2005, Switzer, *et al* 2011) SET is the primary and most potent physiological inhibitor of PP2A, (Neviani and Perrotti 2014, Switzer, *et al* 2011) a major mammalian serine/threonine phosphatase that plays a critical regulatory role in the cell cycle, apoptosis and appropriate deactivation of oncogenic signalling. Decreased activity of PP2A in CLL due to inappropriate SET protein isoform balance may lead to unregulated kinase activity and unchecked growth and replication, thereby permitting progression of the CLL clone. (Ciccone, *et al* 2015)

It is now recognized that there are at least four SET isoforms formed from alternative splicing, with SETA (SET-204) and SETB (SET-201) considered the main protein isoforms. (Adachi, *et al* 1994) To date, investigators have generally focused on the role of total SET in human malignancies without elucidating the impact of relative differences in splice variant expression in disease heterogeneity. Our findings raise questions regarding the shared and independent roles that that the two main splice variants of SET (SETA and SETB) have in oncogenesis and cancer progression, as well as questions regarding the mechanisms that control isoform-specific *SET* expression in CLL. While normal B cells predominantly contained the β isoform of SET protein, we noted in our prior work that both SETA and B protein were expressed in CLL and NHL cells.(Christensen, *et al* 2011) SETA and SETB are both capable of inhibiting PP2A(Saito, *et al* 1999) activity and inhibiting nucleosome acetylation.(Saavedra, *et al* 2017) We are not aware of research identifying the independent functions of the two SET isoforms or the mechanisms that regulate different SET isoform expression in CLL. The relative differences in *SETA* and *SETB* mRNA expression between

CLL patients found in this study highlight the need for ongoing work to elucidate the roles of the different SET isoforms and the factors that control their differential expression.

In our cohorts, we found that increased *SETA* isoform mRNA relative to *SETB* isoform mRNA identifies a high-risk group within the mutated *IGHV* and favourable FISH risk groups (Figure 2). We hypothesize that SET may impact the B-cell receptor (BCR)-mediated kinase pathway signalling, which can be affected by *IGHV* mutation status.(Kipps, *et al* 2017) In UM-*IGHV*, BCR activation can enhance pathways associated with proliferation and cellular survival(Packham, *et al* 2014), and this heightened kinase signalling may be too strong to be negatively regulated by the SET-PP2A axis. Therefore, any relative differences between patients in SET inhibition of PP2A (as reflected in the *SETA/B* mRNA ratio) may not have a large impact in patients with UM-*IGHV*. Conversely, in M-*IGHV* CLL, BCR signalling may activate proliferation pathways to a lesser degree, and this diminished kinase signalling response may be more likely to be modulated by the SET-PP2A axis. Thus, relative changes in SET protein isoforms and subsequent relative changes in PP2A activity could lead to different outcomes based on *IGHV* mutation status.

In summary, we have found that patients with CLL containing relatively higher expression of *SETA* isoform mRNA (high *SETA/B* mRNA ratio) have significantly shorter TTFT and OS compared to those with lower levels, and that this ratio identifies patients with worse clinical outcomes within previously defined CLL favourable risk groups. These results highlight that alternative splicing of *SET* mRNA may be an important oncogenic control mechanism. Additional work is needed to understand the functional differences in SETA and SETB protein isoforms. CLL patients will benefit from a deeper understanding of the function and control of *SET* mRNA isoforms and of alternative splicing in general, in that this knowledge can lead to improved patient counseling and development of novel therapeutics in CLL that modulate epigenetics and/or phosphatase activity.

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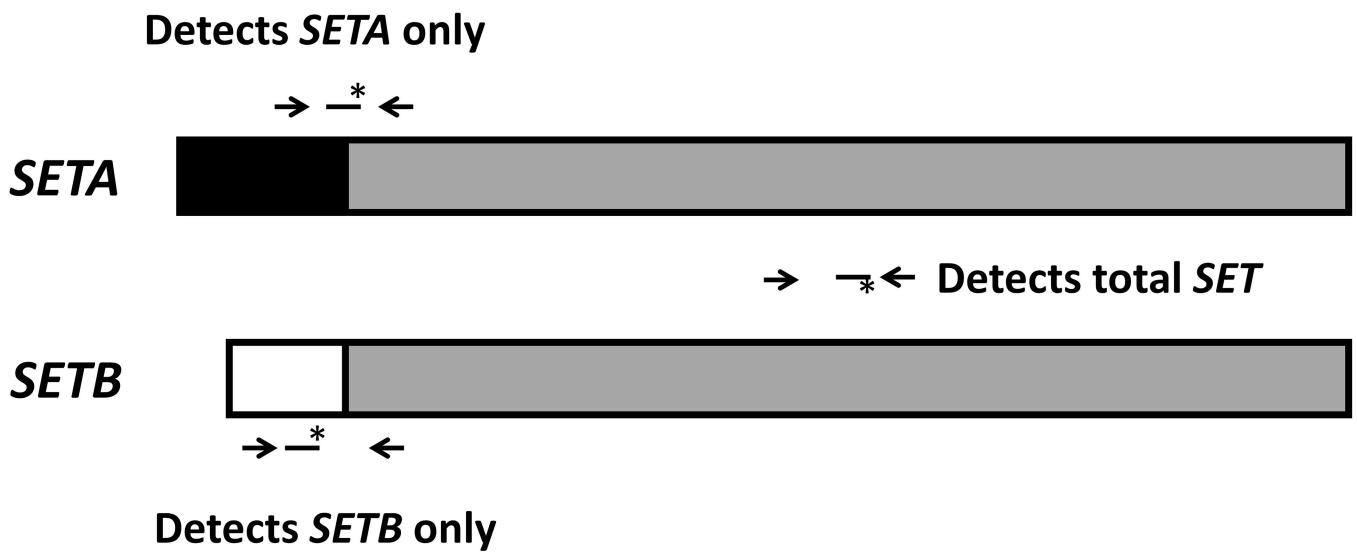

Figure 1: *SETA*, *SETB* and total primers and probes.

The *SETA* forward primer is located within exon 1, which is unique to the alpha form. The *SETB* forward primer is located within exon 2, which is unique to the beta form. The reverse primer for both *SETA* and *SETB* lies within exon 3, which is common to both forms of *SET*. The *SETA* probe lies partially in the alpha exon and partially in the common exon. The *SETB* probe lies entirely in the beta exon. A primer/probe combination was also designed in order to amplify all *SET* mRNA isoforms. The forward primer, the reverse prime, and the probe all lie in the common exon. All primers and probes were purchased from Integrated DNA Technologies (Coralville, Iowa)

SETA forward primer: AGAAGAAACCAAGACCACCTCCTG

Reverse primer for both *SETA* and *SETB*: GTGTTCAATCGCTTCTTGCTGTTC

SETA probe: TGCAGGCTTGCCGAAGAAGGGAGAAA

SETB probe: AAGGAGCTCAACTCCAACCACGACG

Forward primer for all *SET* isoforms: CCA TCT TCG AAG TCC ACC GAA ATC

Reverse primer for all *SET* isoforms: GCC TCT TCC TGC TGG CTT TAT T

Probe for all *SET* isoforms: GGA TTT GAC GAA ACG TTC GAG TCA AAC GCA

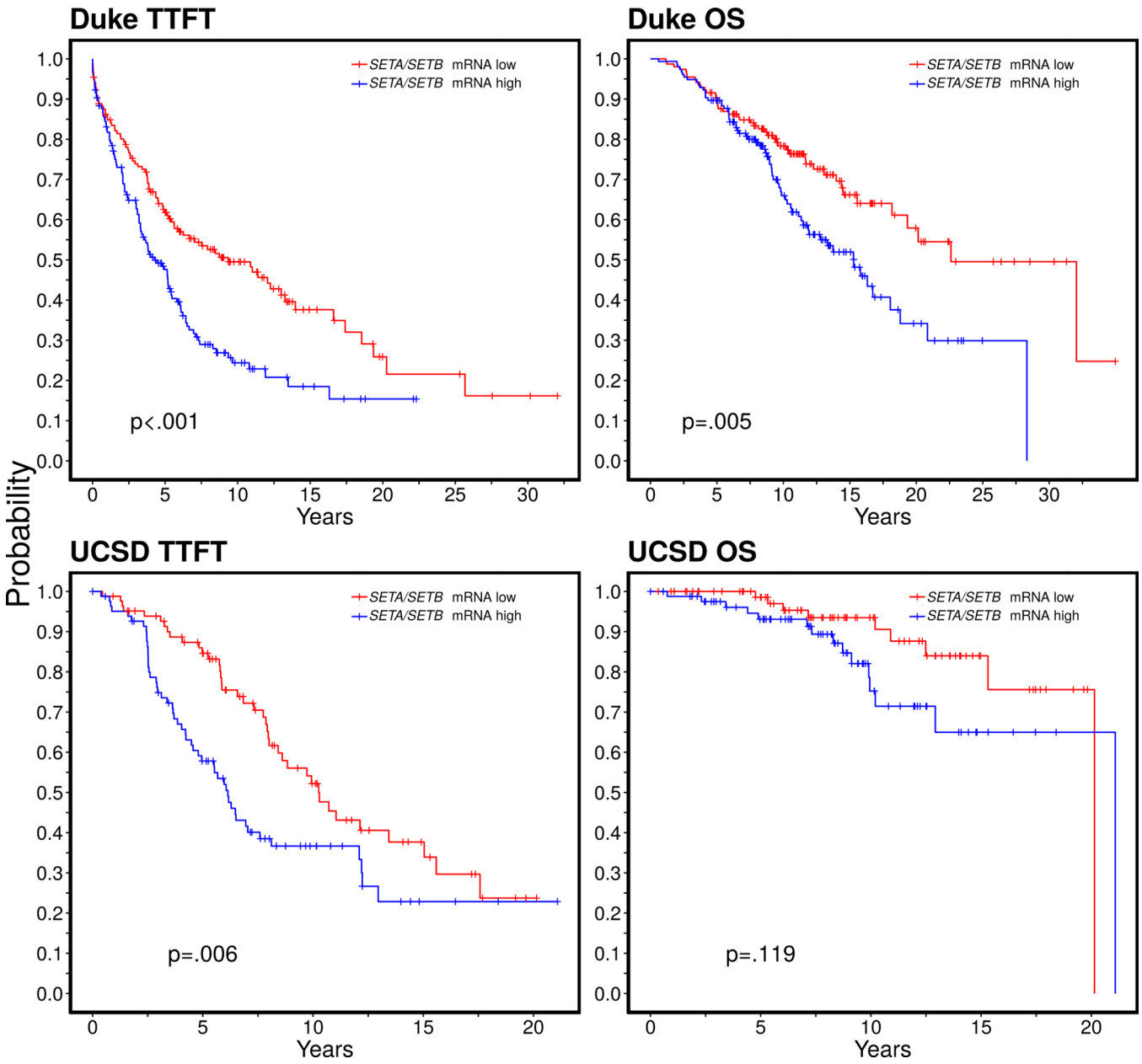

Figure 2. *SETA/B* mRNA ratio analysed relative to time to TTFT and OS for the Duke/DurVAMC cohort (top) and the UCSD cohort (bottom).

This is displayed as a dichotomous variable for illustrative purposes with the respective p-values for significance. High *SETA/B* mRNA ratio was also primarily assessed as a continuous variable in the Duke/DurVAMC cohort in which high *SETA/B* mRNA ratio had significantly shorter TTFT [hazard ratio (HR) 1.2 (1.2–1.3), p=0.001]. Duke/DurVAMC patients with higher *SETA/B* mRNA ratio as a continuous variable also had a significantly shorter OS [HR 1.2 (1.1–1.4), p < 0.001). Analysis of *SET* mRNA in the UCSD patients revealed that a high *SETA/B* mRNA ratio as a continuous variable was also significantly associated with shorter TTFT [HR 1.3 (1.0–1.6), p=0.02]. Duke/DurVAMC:

Duke University or Durham Veteran's Affairs Medical Centers; OS: overall survival; TTFT: time to first treatment; UCSD: University of California San Diego

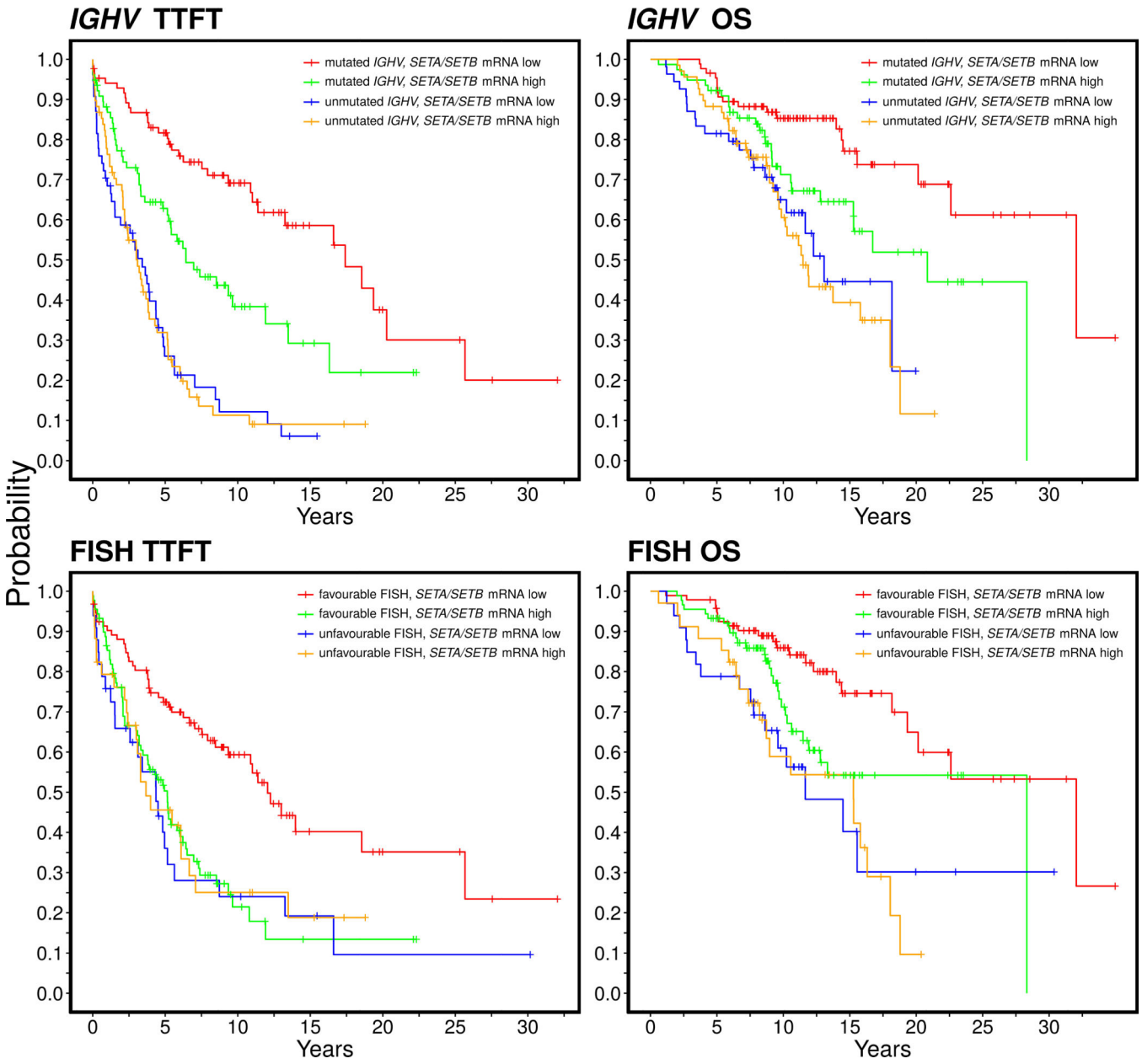

Figure 3. Analysis of the interaction of *SETA/SETB* mRNA ratio with *IGHV* mutation status and FISH group for the Duke/DurVAMC cohort for TTFT and OS.

The figure shows the interaction of *SETA/B* mRNA ratio as a dichotomous variable with *IGHV* mutation status (top) and FISH (bottom) for TTFT (left) and OS (right). As expected, for unfavourable risk UM-*IGHV* patients (blue and yellow lines), there was shorter TTFT compared to M-*IGHV* (red and green lines), and for UM-*IGHV* this poor prognosis appeared independent of the *SETA/B* mRNA ratio. However, within the favourable risk M-*IGHV* patients, those with high *SETA/B* mRNA ratio (red) had a shorter TTFT compared to low *SETA/B* mRNA ratio (green). This interaction between *IGHV* mutation status and *SETA/B* mRNA ratio in TTFT is significant (p=0.01). As expected for patients with unfavourable FISH, defined as any del(17p), del(11q) or three or more abnormalities by

FISH (red and green lines), there was shorter TTFT compared to favourable FISH (blue and yellow lines), and for UM-*IGHV* this poor prognosis appeared independent of the *SETA/B* mRNA ratio. However, within the favourable FISH population, those with high *SETA/B* mRNA ratio (blue) had a shorter TTFT compared to low *SETA/B* mRNA ratio (yellow). This interaction between FISH group and *SETA/B* mRNA ratio in TTFT was significant (p=0.01). Duke/DurVAMC: Duke University or Durham Veteran's Affairs Medical Centers; FISH: fluorescence *in situ* hybridisation; M-*IGHV*: mutated *IGHV*; OS: overall survival; TTFT: time to first treatment; UM-*IGHV*: unmutated *IGHV.*

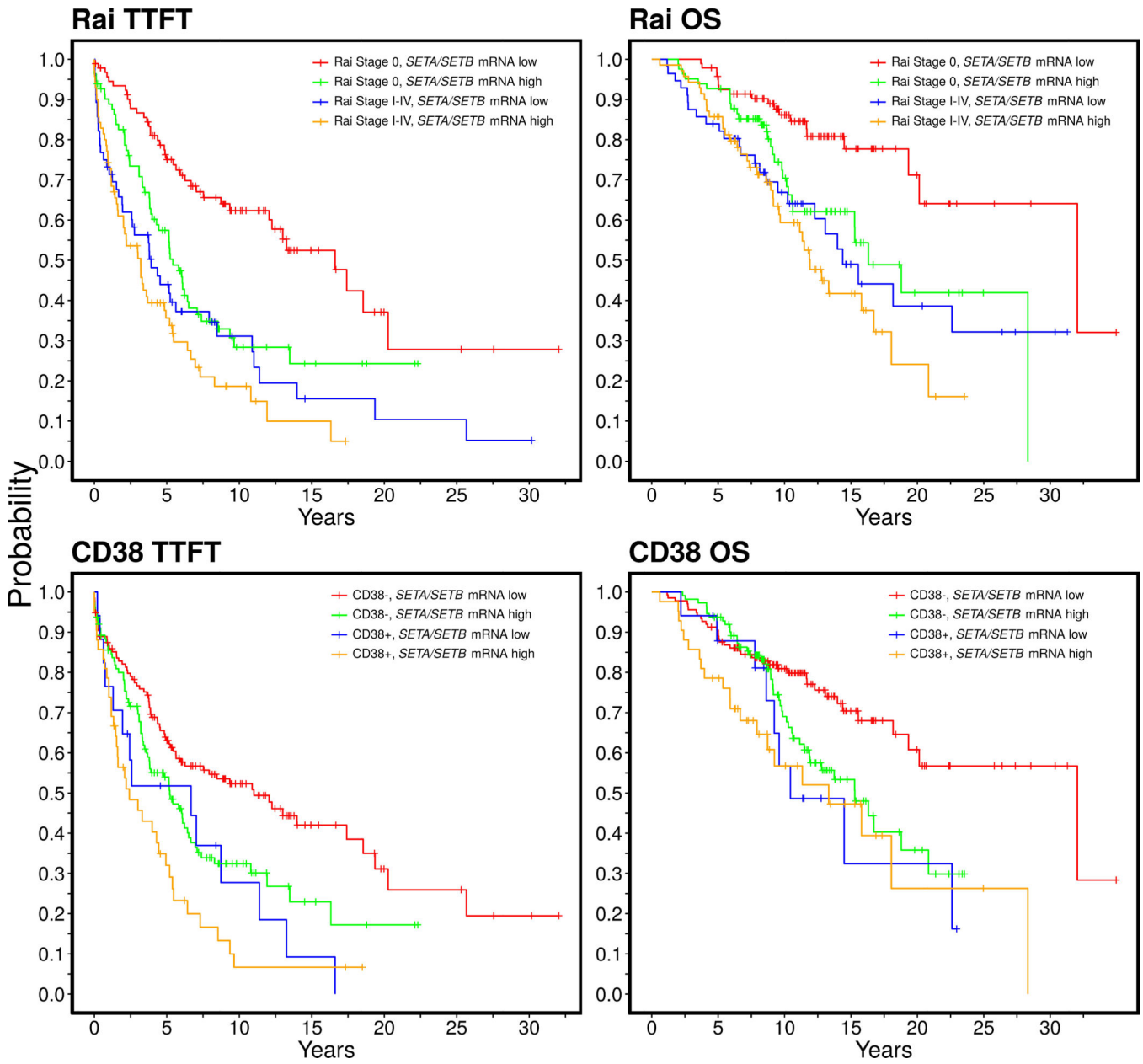

Figure 4. Analysis of the interaction of the *SET* mRNA ratio and Rai stage and CD38 expression for the Duke/DurVAMC cohort for TTFT and OS.

The interaction of *SETA/B* mRNA ratio as a dichotomous variable with Rai stage (top) and CD38 expression (bottom) is displayed for the Duke/DurVAMC cohort for TTFT (left) and OS (right). There was no evidence for significant interaction between *SET* mRNA ratio and either Rai stage or CD38 expression. Duke/DurVAMC: Duke University or Durham Veteran's Affairs Medical Centers; OS: overall survival; TTFT: time to first treatment.

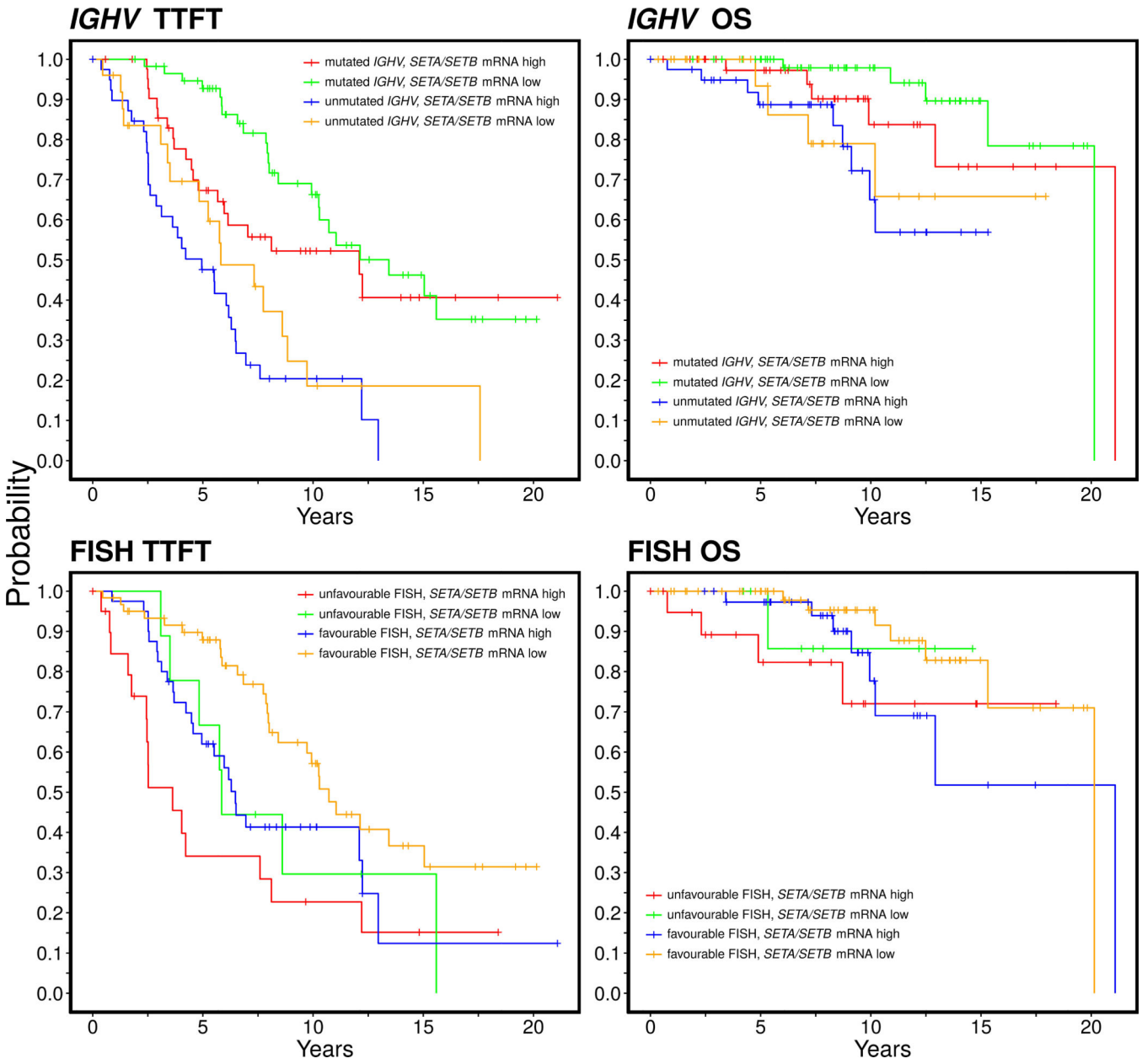

Figure 5. Analysis of the interaction of *SETA/B* mRNA ratio with *IGHV* mutation status and FISH group for the UCSD cohort for TTFT and OS.

The figure shows the interaction of *SETA/B* mRNA ratio as a dichotomous variable with *IGHV* mutation status (top) and FISH (bottom) for TTFT (left) and OS (right). There is no evidence for significant interaction between *SET* mRNA ratio and either *IGHV* mutation status or FISH group. FISH: fluorescence *in situ* hybridisation; M-*IGHV*: mutated *IGHV*; OS: overall survival; TTFT: time to first treatment; UCSD: University of California San Diego; UM-*IGHV*: unmutated *IGHV.*

Table I.

Patient characteristics Duke/DurVAMC and UCSD

	Duke/DurVAMC	UCSD
Number of patients	310	166
Age, years; median, range	68 (33–94)	n/a
Female	32%	40%
Rai Stage (N=302, Duke; N=134 UCSD)	**n (%)**	**n (%)**
0	176 (58%)	81 (60%)
I-IV	126 (42%)	53 (40%)
CD38 positive (N=308, Duke; N=165 UCSD)	60 (19%)	46 (28%)
***IGHV* unmutated** (N=285, Duke; N=166 UCSD)	122 (43%)	65 (39%)
Hierarchical FISH (N=252, Duke; N=131 UCSD)	**n (%)**	**n (%)**
del(17p)	38 (15%)	14 (11%)
del(11q)	34 (14%)	7 (5%)
trisomy 12	33 (13%)	10 (8%)
normal	43 (17%)	32 (24%)
del(13q) alone	104 (41%)	68 (52%)

For hierarchical FISH, groups are listed in order of least favourable (highest risk) to most favourable (lowest risk). del(17p) is presence of any deletion of 17p, alone or in combination with other FISH abnormalities. del(11q) is any deletion of 11q, alone or in combination, but without del(17p). Trisomy 12 is trisomy 12 alone or in combination with del(13q). Normal indicates no FISH abnormalities were detected.

Duke/DurVAMC: Duke University or Durham Veteran's Affairs Medical Centers; FISH: fluorescence *in situ* hybridisation; UCSD: University of California San Diego

Table II.

SET mRNA isoform ratio and established CLL prognostic markers for time to first treatment in Duke/Durham VAMC cohort

	Time to first treatment			Overall survival		
	Median time [years, (95% CI)]	**HR**	**P value**	**Median time [years (95% CI)]**	**HR**	**P value**
SETA/B mRNA ratio						
high	4.3 (3.3–5.5) [n=155]	1.7 (1.3–2.3)	< 0.001	15.3 (11.9–18.8) [n=155]	1.7 (1.2–2.5)	0.005
low	9.3 (5.9–14.0) [n=153]			22.6 (19.3-) [n=154]		
Rai stage						
I-IV	3.3 (2.2–4.9) [n=126]	2.3 (1.7–3.1)	< 0.001	13.1 (11.3–18.0) [n=126]	2.2 (1.5–3.2)	< 0.001
0	8.7 (6.2–13.5) [n=175]			28.3 (19.3-) [n=176]		
CD38 expression						
positive	2.6 (1.6–5.4) [n=59]	2.1 (1.5–2.9)	< 0.001	13.3 (9.2-) [n=59]	2.0 (1.3–3.0)	0.001
negative	6.3 (5.2–10.8) [n=248]			19.3 (16.3-) [n=249]		
IGHV mutation status						
unmutated	3.1 (2.4–3.9) [n=122]	3.3 (2.5–4.6)	< 0.001	11.9 (10.3–18.8) [n=122]	2.5 (1.6–3.7)	< 0.001
mutated	11.9 (9.3–18.5) [n=162]			28.3 (20.8-) [n=163]		
FISH group						
unfavourable	4.4 (3.1–6.1) [n=67]	1.6 (1.1–2.2)	0.01	14.5 (9.6–18.0) [n=67]	2.3 (1.5–3.6)	< 0.001
favourable	7.3 (5.5–10.8) [n=182]			28.3 (19.3-) [n=183]		

CI: confidence interval: CLL: chronic lymphocytic leukaemia; Duke/DurVAMC: Duke University or Durham Veteran's Affairs Medical Centers; FISH: fluorescence *in situ* hybridisation.

Table III.

SET mRNA isoform ratio and established CLL prognostic markers for time to first treatment and overall survival in UCSD cohort

	Time to first treatment			Overall survival		
	Median time [years (95% CI)]	**HR**	**P value**	**Median time [years (95% CI)]**	**HR**	**P value**
SETA/B mRNA ratio						
high	6.1(4.8–8.1) [n=83]	1.8 (1.2–2.7)	0.006	21.1 (12.9-) [n=83]	1.9 (0.8–4.5)	0.12
low	10.3 (8.4–15.6) [n=83]			83, 20.1 (−) [n=83]		
Rai stage						
I-IV	6.8 (5.7–10.3) [n=53]	1.9 (1.2–3.0)	0.007	NA (−) [n=53]	2.3 (0.8–6.5)	0.1
0	1.0 (7.9-) [n=81]			21.1 (−) [n=81]		
CD38 expression						
positive	4.8 (3.5–6.3) [n=46]	2.5 (1.7–3.9)	< 0.001	21.1 (10.2-) [n=46]	1.9 (0.8–4.4)	0.13
negative	10.7 (8.1–15.0) [n=119]			20.1 (−) [n=119]		
IGHV mutation status						
unmutated	5.5 (3.9–6.5) [n=65]	3.3 (2.1–5.0)	< 0.001	NA (10.2-) [n=65]	3.5 (1.5–8.2)	0.002
mutated	12.1 (10.3-) [n=101]			20.1 (20.1-) [n=101]		
FISH group						
unfavourable	4.2 (3.1–8.6) [n=30]	2.0 (1.2–3.3)	0.005	20.1 (15.3-) [n=30]	1.8 (0.6–5.1)	0.3
favourable	10.3(7.9–12.2) [n=101]			101, NA (−) [n=101]		

CI: confidence interval: CLL: chronic lymphocytic leukaemia; FISH: fluorescence *in situ* hybridisation; UCSD: University of California San Diego.