Impact of additional genetic abnormalities at diagnosis of chronic myeloid leukemia for first-line imatinib-treated patients receiving proactive treatment intervention

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Abstract

The *BCR::ABL1* gene fusion initiates chronic myeloid leukemia (CML); however, evidence has accumulated from studies of highly selected cohorts that variants in other cancer-related genes are associated with treatment failure. Nevertheless, the true incidence and impact of additional genetic abnormalities (AGA) at diagnosis of chronic phase (CP)-CML is unknown. We sought to determine whether AGA at diagnosis in a consecutive imatinib-treated cohort of 210 patients enrolled in the TIDEL-II trial influenced outcome despite a highly proactive treatment intervention strategy. Survival outcomes including overall survival, progression-free survival, failure-free survival, and *BCR::ABL1* kinase domain mutation acquisition were evaluated. Molecular outcomes were measured at a central laboratory and included major molecular response (MMR, *BCR::ABL1* ≤0.1%^{IS}), MR4 (*BCR::ABL1* ≤0.01%^{IS}), and MR4.5 (*BCR::ABL1* ≤0.0032%^{IS}). AGA included variants in known cancer genes and novel rearrangements involving the formation of the Philadelphia chromosome. Clinical outcomes and molecular response were assessed based on the patient's genetic profile and other baseline factors. AGA were identified in 31% of patients. Potentially pathogenic variants in cancer-related genes were detected in 16% of patients at diagnosis (including gene fusions and deletions) and structural rearrangements involving the Philadelphia chromosome (Ph-associated rearrangements) were detected in 18%. Multivariable analysis demonstrated that the combined genetic abnormalities plus the EUTOS long-term survival clinical risk score were independent predictors of lower molecular response rates and higher treatment failure. Despite a highly proactive treatment intervention strategy, first-line imatinib-treated patients with AGA had poorer response rates. These data provide evidence for the incorporation of genomically-based risk assessment for CML.

Introduction

Chronic myeloid leukemia (CML) is characterized by the translocation that forms the Philadelphia (Ph) chromosome and the *BCR::ABL1* gene fusion. Targeted therapy with tyrosine kinase inhibitors (TKI)[1-5] inhibits the activated BCR::ABL1 enzyme and, while generally very effective,[6] treatment outcomes remain heterogenous. Up to 15% of patients are resistant to one or more TKI and progression to blast phase (BP) still occurs in approximately

5% of patients.[7] While *BCR::ABL1* kinase domain point mutations remain the primary known mechanism of acquired resistance and direct sequential therapy selection,[7-9] these mutations are identified in only approximately 50% of cases of TKI resistance.[10,11] Kinase domain mutations are not detected at diagnosis in chronic phase (CP) CML and are not the cause of primary resistance, which remains poorly understood. This suggests the existence of alternative resistance pathways.

Unrestrained BCR::ABL1 activity promotes genetic instability and can trigger the development of single nucleotide variants, insertions and deletions.[12-16] We previously performed an integrative genomic analysis in a discovery cohort of CP-CML patients at diagnosis who were specifically selected based on optimal or very poor outcomes, and found that patients who subsequently progressed to BP had a significantly higher frequency of cancer-related gene variants at diagnosis compared with patients who achieved an optimal molecular response (54% *vs.* 16%).[17] Furthermore, all patients tested at BP had one or more cancer gene variants in addition to *BCR::ABL1*, which included gene fusions and gene deletions.[17,18]

Genomic profiling also uncovered novel genomic rearrangements associated with the formation of the Ph chromosome, termed Ph-associated rearrangements.[17] These were structural variants defined as aberrant fusions formed at the time of the Ph translocation, involving rearrangement of genes or sequences on the translocated chromosomes. It is likely that many such variants constitute near-simultaneous genomic rearrangements due to repair of additional double strand breaks related to the formation of the Ph chromosome. They were characterized by sequence fragmentation, non-contiguous deletion, inversion, and imperfect reassembly, likely resulting from genomic 'shattering' and attempted realignment into a mosaic patchwork of genomic fragments.[17,19] These chromoplexy-like rearrangements[20] were highly complex in some patients and have been described in other malignancies to correlate with accelerated cancer evolution.[21] In our discovery cohort, Ph-associated rearrangements were more frequently observed in patients with poor outcomes (33%) compared with those achieving optimal molecular targets (11%).[17] These genomic findings notwithstanding, the true clinical implications of cancer gene variants and Ph-associated rearrangements at the time of CP-CML diagnosis is unknown. Multiple studies have linked cancer gene variants at diagnosis and poor outcome for imatinib-treated patients,[17,22,23] but there has been no systematic evaluation of unselected cohorts to establish the true incidence and clinical risk conferred by genetic abnormalities at diagnosis.

In this study, we investigated the clinical relevance of variants in cancer-related genes that were classified as pathogenic or potentially pathogenic (including gene fusions and deletions), and Ph-associated rearrangements detected at the time of diagnosis in a consecutive cohort of imatinib-treated patients who received rapid treatment intervention that was largely based on time-dependent molecular milestone response values. We show that these genetic abnormalities when detected at the time of diagnosis have a significant impact on treatment response and outcome. The prognostic effect of the genetic abnormalities was independent of the EUTOS long-term survival (ELTS) score, which has already been demonstrated to be discriminative of survival in the TKI era.[24] Our data validate and expand the findings from previous discovery cohort studies where the results suffered from selection bias since patients were selected for sequencing based on their known outcome.[17,22,23]

Methods

Ethics approval was obtained from the local Institutional Review Board and the study was performed in accordance with the Declaration of Helsinki.

Patients

The total cohort of 210 adult CP-CML patients enrolled in the Australasian Leukemia and Lymphoma Group CML9 study (TIDEL-II, ACTRN12607000325404) were investigated.[25] Patients were treated with first-line imatinib 600 mg daily with active intervention: either dose escalation or nilotinib switch for lack of achievement of time-dependent molecular milestones. Imatinib was also dose escalated for subtherapeutic trough levels (<1000 ng/mL) at day 22. The molecular milestones were *BCR::ABL1* transcript ratios of ≤10%, ≤1%, and ≤0.1%^{IS} at 3, 6 and 12 months, respectively, similar to those subsequently adopted by the European LeukemiaNet (ELN) as optimal time-dependent responses to TKI therapy.[26] Patients were also switched to nilotinib for imatinib intolerance or loss of response, defined as at least one of the following: loss of confirmed complete hematologic response or major cytogenetic response, cytogenetic clonal evolution, a confirmed >5-fold increase in *BCR::ABL1* ratio from nadir to a level >0.1% resulting in loss of MMR, a greater than 2-fold increase from nadir in *BCR::ABL1* ratio to a level of >10%, detection of >50% mutant *BCR::ABL1*, or disease transformation to accelerated phase (AP) or BP.[25] The method of TKI compliance assessment is included in the Patient Compliance Assessment available in the *Online Supplementary Appendix*.

Next-generation sequencing

Following appropriate approval from the local institutional review board, diagnostic blood samples were sequenced using a customized RNA-based capture panel of 126 genes[27] (*Online Supplementary Table S1*), or whole exome and/or

whole transcriptome sequencing, as previously described.[17] We previously demonstrated that total RNA is suitable for detecting multiple different types of variants, including single nucleotide variants (SNV), small insertions and deletions (indels), fusions (genomic and transcripts), and gene deletions and their corresponding genomic breakpoints from pre-spliced RNA.[17,27] Identified variants were reviewed using strict criteria, as detailed previously.[17] Of the variants that met our criteria for damaging, we only included those considered as likely pathogenic or pathogenic using the criteria set out in joint consensus standards for the classification of somatic variants.[28,29] Further details on sequencing methods are provided in the *Online Supplementary Methods*.

Statistical analysis

We assessed the association between specific baseline variables and molecular response and outcome by four years, including additional genetic abnormalities (AGA), ELTS clinical risk scores, age, sex, and *BCR::ABL1* transcript type. AGA were assessed both in aggregate and in separate categories (cancer gene variants and Ph-associated rearrangements). Variables with *P* values <0.05 using univariate analysis were assessed in bi/multivariable analysis for the relevant outcome. Confounding variables that were significant using univariate testing required separate assessment on bi/multivariable analysis, such as age and ELTS. The Bayesian information criterion indicated the best multivariable model for selection. Variables that emerged as significant on univariate analysis were assessed for independence utilizing the variance inflation factor to test for multicollinearity. Multiple comparison correction was performed using the Benjamini-Hochberg Method.[30] Overall survival (OS) and failure-free survival (FFS) were calculated through Kaplan-Meier estimates and log-rank tests. Patients were censored at study withdrawal or completion of follow-up visits. Independent predictors of OS and FFS were tested using Cox regression analysis. Failure events were as currently defined by the ELN,[7] which included failure to achieve time-dependent molecular milestones, acquisition of *BCR::ABL1* kinase domain mutations, AP/BP and death by any cause. The cumulative incidence of *BCR::ABL1* kinase domain mutation acquisition and disease progression to AP or BP were assessed through Fine-Gray modeling, as were the molecular outcomes of major molecular response (MMR, *BCR::ABL1* ≤0.1%^{IS}), MR4 (*BCR::ABL1* ≤0.01%^{IS}), and MR4.5 (*BCR::ABL1* ≤0.0032%^{IS}). Schoenfeld residuals were used to test the assumption of proportional hazards. Death not related to AP/BP and progression to AP/BP were considered competing risks. Statistical analysis was performed using GraphPad Prism 8.0.0 and R version 4.1.2.

This trial was registered on www.anzctr.org.au with the identifier ACTRN12607000325404.

Results

Diagnostic samples were successfully sequenced for 200 of the 210 patients (95%) with characteristics of these patients summarized in Table 1. The median follow-up of patients was 37 months (range 2.5-48 months). RNA capture panel sequencing was available for 188 patients, and 12 patient samples were sequenced using whole-exome sequencing and/or whole transcriptome RNA-Seq.[17] Data for 17 samples have been previously reported.[17] Six samples were sequenced using RNA-seq and repeated using the RNA capture panel to demonstrate concordance, and have been reported previously.[27] Results were not available for nine patients due to RNA degradation or inadequate sample. *BCR::ABL1* fusion transcripts were detected in all samples and the genomic breakpoints that generated the *BCR::ABL1* transcript were detected in 85% of patients (*Online Supplementary Table S2*).

Genomic findings

At the time of diagnosis, 40 SNV and indels in cancer genes were detected in 33 patients (16%) across ten genes (Figure 1A). One of these patients also had a gene fusion involving *IKZF1* and a deletion involving *RUNX1*, and rapidly progressed to BP (see Figure 2). Ph-associated rearrangements were identified in 36 patients (18%). The complexity of these rearrangements is detailed in Figure 1B, *Online Supplementary Figure S1*, and in the *Online Supplementary Results*. There was no significant difference in the age of patients without AGA (median age 50 years, range 17-81 years) compared to those with cancer gene variants (median age 51 years, range 23-71 years) or Ph-associated rearrangements (median age 45 years, range 17-79 years). Overall, 121/200 patients (61%) expressed the reciprocal *ABL1::BCR* transcript. Ph-rearrangements were strongly associated with the absence of *ABL1::BCR*. Of 36 patients with Ph-associated rearrangements, only one expressed *ABL1::BCR*. The rearrangement for this patient comprised a genomic inversion between *BCR* and *ABL1* that was immediately adjacent to the standard *BCR::ABL1* genomic breakpoint. Lack of a reciprocal transcript in 97% of patients with a Ph-associated rearrangement is consistent with deletion or rearrangement of sequence adjacent to the *ABL1* and/or *BCR* breakpoints in many cases. *Online Supplementary Table S2* details the predicted size of deletions based on the genomic location of the fusion partners of patients with Ph-associated rearrangements. Eight patients had both cancer gene variants and Ph-associated rearrangements, accounting for 4.5% of the sequenced population. Collectively, 61 patients (31%) harbored AGA. The data for individual patients are detailed in *Online Supplementary Table S2*.

ASXL1 was the most frequently mutated gene at diagnosis (Figure 1A) with 20 variants observed in 18 of 200 patients

Table 1. Patients' characteristics.

Patients' characteristics	All patients with sequencing data (N=200)	Sub-group breakdown			
	Number (%) or median [range]	No AGA (N=139)	Cancer-gene variants alone (N=25)	Ph-associated rearrangements alone (N=28)	Both cancer-gene variants and Ph-associated rearrangements (N=8)
Male	113 (57)	77 (55)	17 (68)	15 (54)	4 (50)
Age at diagnosis, yrs	50 [17-81]	50 [17-81]	51 [23-71]	45 [17-79]	47 [24-55]
Transcript					
e13a2	82 (41)	59 (42)	5 (20)	16 (57)	2 (25)
e14a2	80 (40)	56 (40)	11 (44)	9 (32)	4 (50)
Both e13a2 and e14a2	36 (18)	24 (17)	8 (32)	3 (11)	1 (13)
Other*	2 (1)	0	1 (4)	0	1 (13)
ELTS					
Low	126 (63)	92 (66)	15 (60)	15 (54)	4 (50)
Intermediate	46 (23)	27 (19)	8 (32)	9 (32)	2 (25)
High	19 (9)	13 (9)	1 (4)	3 (11)	2 (25)
N/A	9 (4)	7 (5)	1 (4)	1 (4)	0
Progressed to BP	8 (4)	4 (3)	2 (8)	1 (3)	1 (13)
BCR::ABL1 kinase domain mutation acquisition	12 (6)	3 (2)	2 (8)	5 (17)	2 (25)
Nilotinib switch for time-dependent suboptimal molecular response	53 (26)	28 (20)	9 (36)	12 (41)	4 (50)

AGA: additional genetic abnormalities; Ph-associated: Philadelphia chromosome-associated; yrs: years; ELTS: EUTOS long-term survival score; BP: blast phase; N/A: not available. *Included one patient with e1a2 and one patient with e13a3 *BCR::ABL1* transcripts.

(9.0%). Further details of patients with mutated *ASXL1* are provided below. Other genes mutated in multiple patients were *RUNX1*, *DNMT3A* and *TET2*. Two patients had *BCORL1* variants at diagnosis and both progressed to BP (see Figure 2). Four patients had evidence of clonal hematopoiesis of indeterminate potential with somatic variants affecting *DNMT3A*, *ASXL1* or *TET2* that were detected in both the diagnosis and remission samples, and therefore likely predated the acquisition of the Ph chromosome. Three of these four patients achieved an optimal response while one failed therapy and acquired a *BCR::ABL1* kinase domain mutation; this patient also had a distinct somatic *ASXL1* variant exclusively within the leukemic clone at diagnosis. Variants that affected RNA splicing were also identified by this methodology (*Online Supplementary Figure S2A, B*).

We investigated the clinical impact of the AGA on response and outcome. The presence of any genetic abnormality (cancer gene variant or Ph-associated rearrangement) predicted inferior FFS and molecular responses.

Overall and progression-free survival

There were eleven deaths by the end of the 4-year follow-up period with an OS of 94% (95% CI: 87.9-97.9%) (*Online Supplementary Figure S3A*). Six patients succumbed to non-CML-related causes of death (4 cardiac-related deaths, one cerebrovascular accident, one secondary to infection, and one unknown cause of death). Eight patients progressed to BP (5 myeloid and 3 lymphoid BP) and no patient progressed to AP, with a cumulative incidence of progression of 6% (95% CI: 2.3-12.0%) (*Online Supplementary Figure S3B*). Of the eight patients that progressed to BP, five died. No variable predicted for progression to BP or OS by Cox regression analysis.

Failure-free survival

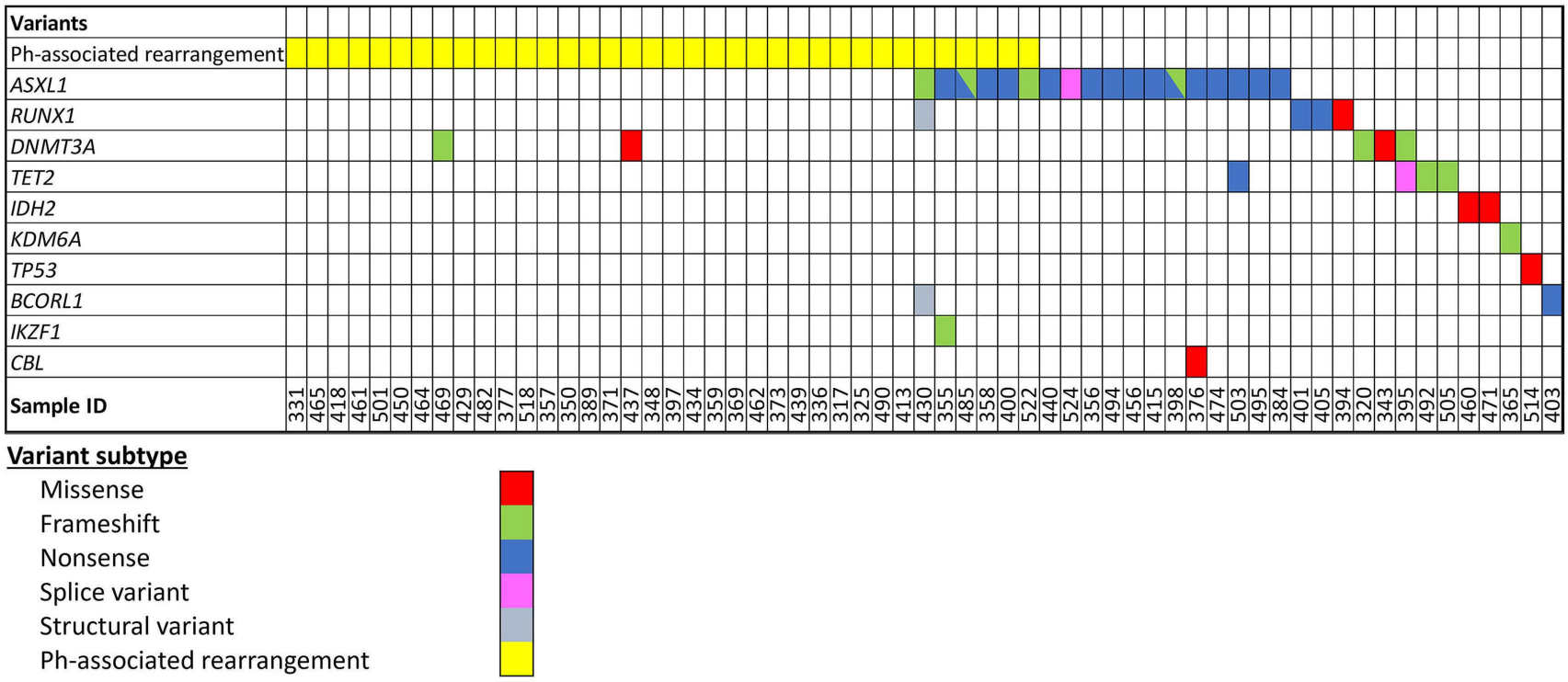
The 4-year estimate of FFS, as defined by the ELN, was 77% (95% CI: 70.2-82.3%) (*Online Supplementary Figure S3C*). Univariate analysis confirmed that AGA at diagnosis predicted for inferior FFS (69% *vs.* 80%, *P*=0.03) (Figure 3A). The ELTS score was also a predictor of FFS (*Online Supplementary Figure S4A*). No other baseline variable predicted FFS. Multivariable analysis using Cox regression analysis confirmed that the only independent predictors of FFS were the presence of AGA at diagnosis (*P*=0.04) and the ELTS score (*P*=0.001) (Table 2).

Acquisition of *BCR::ABL1* kinase domain mutations

Twelve patients acquired *BCR::ABL1* kinase domain muta-

A

Oncoplot of additional genetic abnormalities identified



B

Circos plot of Ph-associated rearrangements

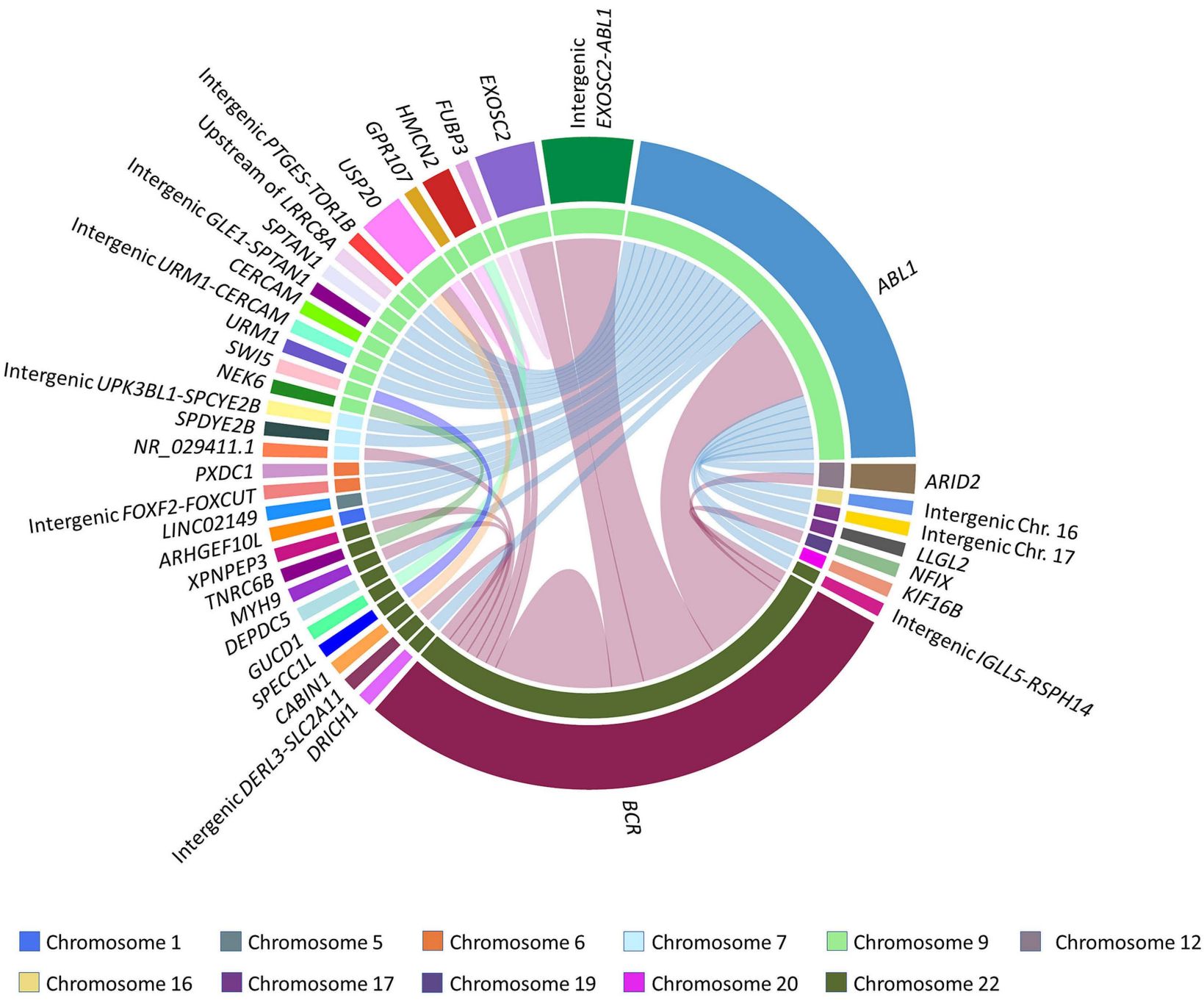


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Figure 1. The genomic findings of the TIDEL-II cohort at diagnosis. (A) Oncoplot of additional genetic abnormalities identified at diagnosis. (B) The predominant Philadelphia chromosome (Ph)-associated rearrangements for individual patients based on the fusion read counts are shown. The diversity of the partner genes (outer circle) involved in the Ph-associated rearrangements is illustrated. Color of the inner circle corresponds to the chromosome location of the involved gene. Width of the ribbon correlates with the frequency of co-occurrence of the fusion partner. All events were in addition to the primary *BCR::ABL1* transcript. The complete list of Ph-associated rearrangements is documented in *Online Supplementary Table S2*. Fusions between *BCR* and *ABL1* in the Circos plot represent inversions.

tions with a cumulative incidence of 6% by four years (95% CI: 3.3-10.2%) (*Online Supplementary Table S2* and *Online Supplementary Figure S3D*). The kinase domain mutations were acquired at a median of 323 days (range 85-722 days) from TKI commencement. By univariate analysis, having an AGA at diagnosis predicted for the acquisition of *BCR::ABL1* kinase domain mutations by four years (15% *vs.* 2%, *P*<0.001) (Figure 3B). Notably, Ph-associated rearrangements were strongly associated with the acquisition of kinase domain mutations (*P*<0.001) (*Online Supplementary Table S3*). While the ELTS score was a significant predictor of the acquisition of kinase domain mutations on univariate analysis (Table 2), the false discovery rate corrected for this. AGA at diagnosis remained the only predictor for the acquisition of *BCR::ABL1* kinase domain mutations.

Molecular responses by four years

Inferior achievement of MMR, MR4 and MR4.5 was associated with the presence of AGA at diagnosis. MMR at 12 months of TKI therapy is an optimal response[7] and lack of MMR at the 12-month milestone was a trigger for treatment intervention for the study cohort. By 12 months, the cumulative rate of MMR was 61% (95% CI: 54-67%) for the study population (*Online Supplementary Figure S3E*). The rate of MMR by 12 months in patients with AGA was significantly lower than those without these events (46% *vs.* 67%, *P*=0.005) (Figure 3C). The overall cumulative rate of MMR by four years was 82% (95% CI: 76-87%). Univariate analysis (Table 2) demonstrated that AGA at diagnosis predicted for an inferior cumulative incidence of MMR by four years compared with patients without these events (72% *vs.* 86%, *P*=0.007) (Figure 3C). ELTS was a predictor for MMR by four years: 87%, 73% and 68% for low-, intermediate- and high-risk groups, respectively (*P*<0.001) *(Online Supplementary Figure S4B*). To avoid collinearity, age and ELTS were assessed separately with AGA at diagnosis using bivariable analysis. The strongest model contained both AGA (*P*=0.025) and the ELTS score (*P*<0.001) (Table 2).

By four years, 59% (95% CI: 51-65%) and 43% (95% CI: 35-50%) of evaluated patients achieved MR4 and MR4.5, respectively, by Fine-Gray modeling (*Online Supplementary Figure S3F, G*). AGA were associated with a significantly lower incidence of MR4 (37% *vs.* 67%, *P*=0.001) and MR4.5 (27% *vs.* 49%, *P*=0.03) (Figure 3D, E). The strongest multivariable analysis demonstrated that AGA and the ELTS score were the only independent predictors of MR4 (AGA *P*=0.004 and ELTS *P*<0.001) and MR4.5 (AGA *P*=0.03 and ELTS *P*=0.02) (Table 2).

Combining additional genetic abnormalities and EUTOS long-term survival clinical risk score to predict molecular outcomes

As AGA and ELTS were the only independent predictors of FFS, MMR, MR4 and MR4.5, the impact of combining the two variables for the prediction of these outcomes was assessed. The intermediate-risk and high-risk groups tracked together for each of the assessed outcomes, and, as they were not statistically different from each other, the two groups were combined to increase sample size.

AGA were detected in 34 of 126 patients (27%) with low ELTS score but did not predict FFS (Figure 4A). For the combined intermediate- (n=46) / high-risk (n=19) patients, 25 of 65 (38%) had AGA. FFS was significantly inferior for intermediate-/high-risk patients with AGA compared with those without (46% *vs.* 71%, respectively, *P*=0.04) (Figure 4B). With respect to MMR achievement, the impact of combining AGA and ELTS was evident across the low-risk and intermediate-/high-risk groups (Figure 4C, D). The cumulative incidence of MMR by 12 months for low-risk ELTS patients with AGA was 50% (95% CI: 32-66%) compared with 77% (95% CI: 67-85%) for low-risk patients without AGA (*P*=0.003) (Figure 4C). For intermediate- / high-risk ELTS patients, the respective values were 20% (95% CI: 7-31%) compared with 43% (95% CI: 32-58%) for those without (*P*=0.03) (Figure 4D). Notably, none of the 19 patients in the high-risk ELTS group with an AGA achieved MMR by 12 months. The statistical difference observed at 12 months was not observed at 48 months and was likely due to patients with AGA having slower achievement of MMR, but eventually reaching the target milestone.

Inferior MR4 and MR4.5 were predicted by the presence of AGA among the ELTS risk groups. MR4 in low-risk ELTS patients with AGA was lower compared with those without AGA (48% *vs.* 72% by four years, *P*=0.025) (Figure 4E). The respective values for intermediate- / high-risk ELTS patients were 17% *versus* 59% (*P*=0.011) (Figure 4F). No patient with high-risk ELTS with AGA at diagnosis achieved MR4 by four years. The cumulative incidence of MR4.5 by four years in the low-risk ELTS group with AGA was 36% *versus* 55% in those without AGA (*P*=0.053) (Figure 4G). The respective values for intermediate- / high-risk ELTS

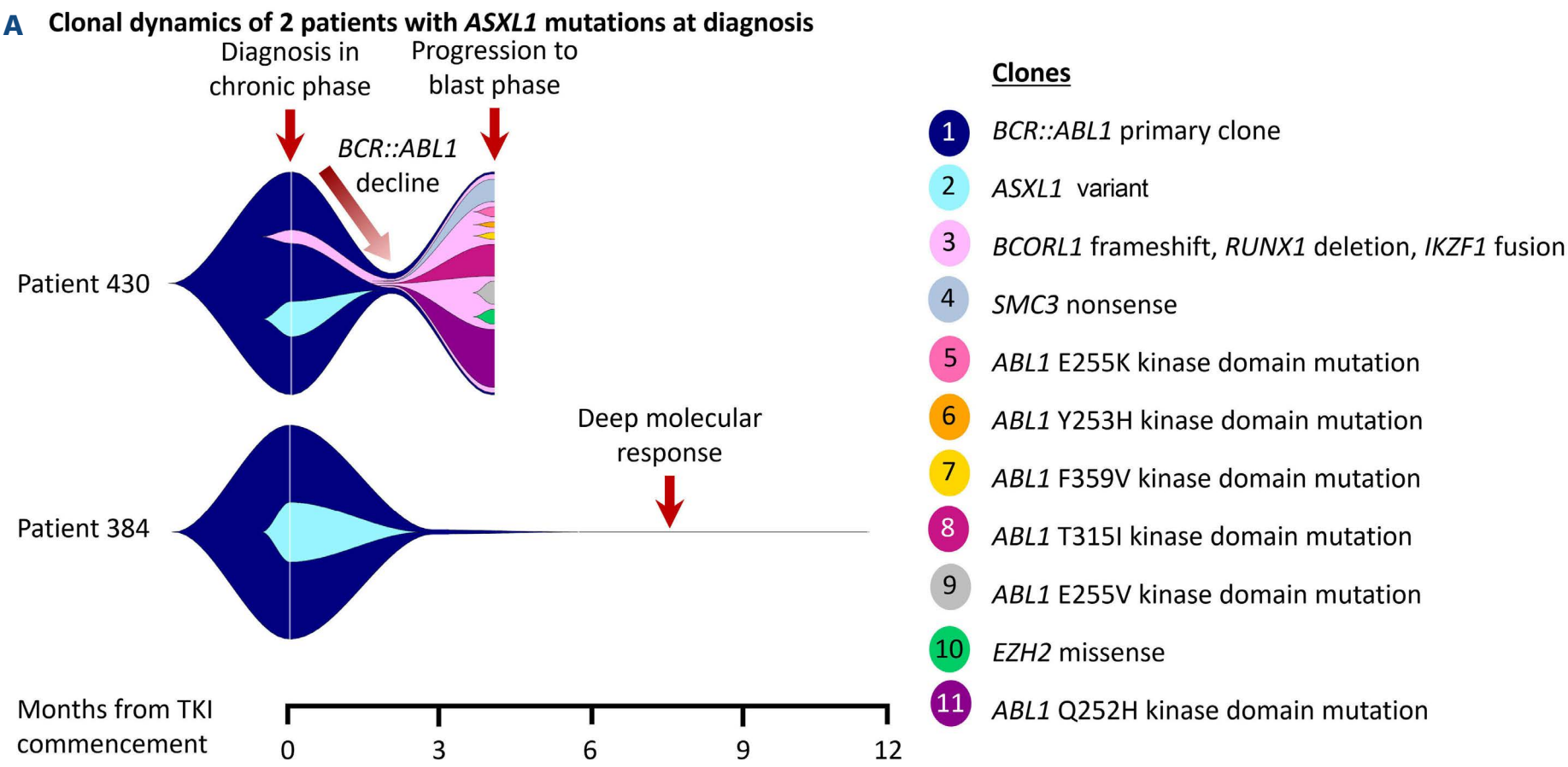

Figure 2. The clonal dynamics of two patients with *ASXL1* variants at diagnosis illustrated by fishplots. (A) Patient 430 had a Philadelphia chromosome (Ph)-associated rearrangement in addition to the *BCR::ABL1* clone (dark blue) at diagnosis. The Ph-associated rearrangement involved an inversion between *ABL1* exon 1 and an intergenic region on chromosome 17q12. In addition, two mutant subclones were detected at diagnosis. One clone contained an *ASXL1* frameshift variant (light blue) and the second subclone harbored a *BCORL1* frameshift variant, a 34 Kb *RUNX1* deletion, and an *IKZF1::IGKV3-7* fusion (represented in pink). With the decline of *BCR::ABL1* in response to imatinib, the subclones were reduced and the *ASXL1* clone became undetectable. However, the second clone expanded and evolved to lymphoid blast phase at 4 months of imatinib. Several independent subclones arose from the original *BCORL1/RUNX1/IKZF1* subclone, including an *SMC3* nonsense variant, an *EZH2* missense variant, and six *ABL1* kinase domain mutations. The second patient (384) harbored a single *ASXL1* nonsense variant at diagnosis. A rapid deep molecular response was achieved and the *ASXL1* subclone became undetectable. The optimal response was maintained. (B) Integrative Genomics Viewer (IGV) screenshot of amino acids 252-255 of *ABL1* of patient 430, demonstrating that the Q252H, Y253H, E255K and E255V kinase domain mutations were on separate reads, which indicates that these mutations were in separate clones and were not compound mutations. Only a proportion of each of the mutant reads is shown. TKI: tyrosine kinase inhibitor.

patients were 9% *versus* 24% (P=0.12) (Figure 4H). Again, among the high-risk ELTS patients, none with AGA achieved MR4.5 by four years.

Impact of additional genetic abnormalities on molecular response for patients who switched to nilotinib

Trial-specific criteria for imatinib dose escalation or switch to nilotinib occurred for time-dependent suboptimal molecular response,[25] which was more proactive than in current treatment guidelines. Patients also switched to nilotinib for loss of response or imatinib intolerance. Seventeen patients dose escalated imatinib to 800 mg daily due to failure to meet trial-defined targets, which included six patients with AGA. TKI switch to nilotinib due

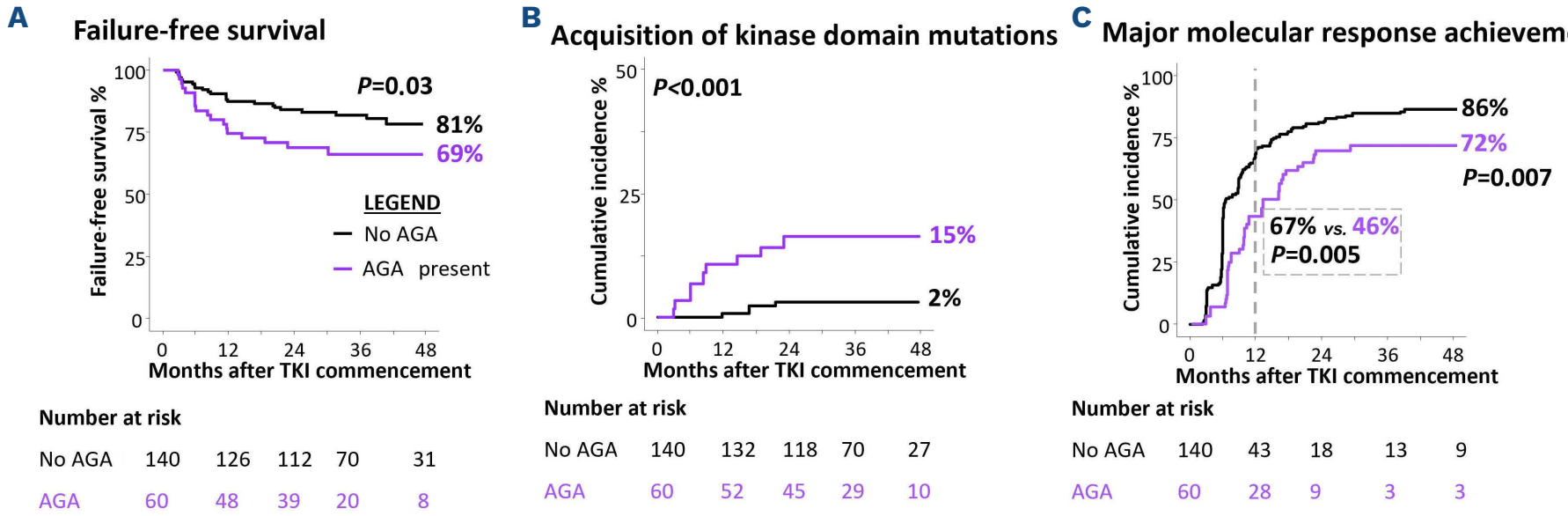

Figure 3. Additional genetic abnormalities at diagnosis were an independent predictor of specific outcomes and molecular responses. The graphs show the outcomes and responses according to additional genetic abnormalities (AGA) status at diagnosis. (A) Kaplan-Meier estimates of failure-free survival. (B) Cumulative incidence of the acquisition of *BCR::ABL1* kinase domain mutations. (C) Cumulative incidence of major molecular response. (D) Cumulative incidence of MR4. (E) Cumulative incidence of MR4.5. The number at risk table is included below the relevant graphs. TKI: tyrosine kinase inhibitor.

to failure to reach a subsequent milestone occurred in 14/16 evaluable patients. One patient switched to nilotinib due to intolerance of the higher imatinib dose, whereas two patients with AGA achieved the subsequent molecular milestone. All that can be concluded from these limited data is that dose escalation of imatinib based on failure criteria had limited response, irrespective of the presence of AGA or not. We assessed whether switch to nilotinib for any reason other than imatinib intolerance could rescue the inferior responses associated with AGA at diagnosis. Fifty-three patients switched to nilotinib for reasons other than imatinib intolerance at a median of 189 days (range 85-678 days) (*Online Supplementary Table S4*), and the switch occurred more often in patients with AGA than patients without (40% *vs.* 20%, respectively, *P*=0.004).

The cumulative incidence of MMR by four years for the 53 patients who switched to nilotinib for reasons other than intolerance was numerically lower if AGA were present at diagnosis, although this did not reach significance (59% *vs.* 74%, *P*=0.11) (Figure 5A). Fourteen of the 53 patients switched to nilotinib at 12 months for lack of MMR, and 11 of these 14 achieved MMR following nilotinib switch. The cumulative incidence of MR4 by four years was significantly lower if AGA were present at diagnosis (13% *vs.* 47%, *P*=0.003) (Figure 5B). Likewise, the 4-year cumulative incidence of MR4.5 was only 6% if AGA were present compared with 24% for patients without AGA (*P*=0.046) (Figure 5C). There was no significant difference in the cumulative incidence of 12-month MMR, 4-year MR4 or MR4.5 for the 24 patients who switched to nilotinib for imatinib intolerance, irrespective of the presence of AGA (*data not shown)*.

Outcome for patients with mutated *ASXL1*

ASXL1 was the most frequently mutated cancer-associated gene at diagnosis. Eighteen patients had frameshift, nonsense or splice site *ASXL1* variants. These 18 patients had an inferior 4-year FFS compared with patients with other AGA or without any AGA (60% *vs.* 75% *vs.* 81%, respectively, *P*=0.045) (Figure 6A). Evolution to BP was only observed for one patient with mutated *ASXL1* at diagnosis, although the *ASXL1* mutant was noticeably absent at progression (Figure 2). Notably, four of the 18 patients (22%) acquired *BCR::ABL1* kinase domain mutations, compared with 12% of patients with other AGA and 2% of patients without any AGA (*P*<0.001) (Figure 6B). None of the other molecular outcomes were significantly different between patients with mutant *ASXL1*, other AGA, or those with no AGA. The most frequently detected *ASXL1* variant in our

cohort was a nonsense variant at R693, which was identified in six of the 20 patients with *ASXL1* variants (*Online Supplementary Table S2*). This is among the most frequently reported *ASXL1* variant. Only two patients had the *ASXL1* mutant G646Wfs*12 at the time of diagnosis in our cohort, which is the most frequently reported *ASXL1* variant.

Interestingly, not all patients with *ASXL1* mutants at diagnosis had an adverse outcome. Figure 2 contrasts the dynamics of clonal expansion and disappearance of two patients with responses spanning either end of the therapeutic spectrum. At diagnosis, patient 430 had a Ph-associated rearrangement as part of the *BCR::ABL1* clone, plus two mutant subclones; one harbored an *ASXL1* frameshift variant and the other harbored a *BCORL1* frameshift variant, an *IKZF1* fusion and a *RUNX1* deletion. The patient rapidly progressed to lymphoid BP at four months after an initial response to imatinib, and additional

Table 2. Cox regression analysis demonstrating the univariate and multivariable analysis for each 4-year outcome.

Outcome measure	Variable	CI of event (%)	Univariate analysis			Multivariable analysis		
			Sub-distribution /Hazard ratio (95% CI)*	*P*	FDR	Sub-distribution /Hazard ratio (95% CI)*	*P*	
Failure-free survival (N of events = 41/200)	No AGA *vs.* AGA	80 *vs.* 69	1.76 (1.04-3.14)	0.02	0.038	1.82 (1.57-3.43)	0.04	
	ELTS Low Intermediate High	 86 65 58	 - 2.60 (1.23-4.73) 2.78 (1.43-7.92)	 - 0.01 0.005	 0.01	 - 2.50 (1.24-5.04) 3.51 (1.51-8.14)	 - 0.01 0.003	 Wald test: *P*=0.001
	Age (<63 *vs.* ≥63 yrs)	74 *vs.* 87	0.45 (0.17-1.21)	0.14	0.28	-	-	-
	Sex (F *vs.* M)	82 *vs.* 78	1.20 (0.44-1.55)	0.56	0.70	-	-	-
	Transcript type e14a2 e13a2 e14a2/e13a2	 80 80 78	 - 1.01 (0.50-2.03) 1.12 (0.48-2.63)	 - 0.97 0.78	 0.96	 -	 -	 -
BCR::ABL1 KD mutation (N of events = 12/200)	No AGA *vs.* AGA	2 *vs.* 15	7.69 (2.04-25)	<0.001	0.015	7.69 (2.04-25)	<0.001	
	ELTS Low Intermediate High	 3 16 5	 - 1.65 (1.53-1.77) 1.13 (0.98-1.91)	 - 0.008 0.06	 0.073	 -	 -	 -
	Age (<63 *vs.* ≥63 yrs)	10 *vs.* 5	1.01 (0.97-1.04)	0.71	0.70	-	-	-
	Sex (F *vs.* M)	5 *vs.* 7	1.55 (0.19-2.14)	0.47	0.59	-	-	-
	Transcript type e14a2 e13a2 e14a2/e13a2	 10 4 3	 - 0.35 0.27	 - 0.12 0.22	 0.20	 -	 -	 -
MMR (N of events = 163/200)	No AGA *vs.* AGA	86 *vs.* 72	0.62 (0.43-0.87)	0.007	0.016	0.70 (0.47-0.94)	0.025	
	ELTS Low Intermediate High	 87 73 68	 - 0.53 (0.37-0.76) 0.40 (0.26-0.63)	 - <0.001 <0.001	 0.005	 - 0.54 (0.38-0.78) 0.41 (0.25-0.66)	 - 0.003 0.001	 Wald test: *P*<0.001
	Age (<63 *vs.* ≥63 yrs)	76 *vs.* 95	2.26 (1.52-3.35)	0.01	0.017	-	-	-
	Sex (F *vs.* M)	87 *vs.* 86	1.16 (0.85-1.60)	0.36	0.45	-	-	-
	Transcript type e14a2 e13a2 e14a2/e13a2	 81 79 83	 - 0.78 (0.55-1.09) 0.92 (0.58-1.44)	 - 0.14 0.7	 0.75	 -	 -	 -

Continued on following page.

Outcome measure	Variable	CI of event (%)	Univariate analysis			Multivariable analysis		
			Sub-distribution /Hazard ratio (95% CI)*	*P*	FDR	Sub-distribution /Hazard ratio (95% CI)*	*P*	
MR4 (N of events = 117/200)	No AGA *vs.* AGA	67 *vs.* 37	0.48 (0.31-0.76)	0.001	0.005	0.51 (0.32-0.82)	0.004	
	ELTS Low Intermediate High	 66 42 41	 - 0.47 (0.30-0.74) 0.45 (0.26-0.79)	 - <0.001 0.002	 0.020	 - 0.51 (0.33-0.81) 0.43 (0.25-0.75)	 - 0.004 0.003	 Wald test: *P*<0.001
	Age (<63 *vs.* ≥63 yrs)	53 *vs.* 78	1.86 (1.17-2.94)	0.032	0.53	-	-	-
	Sex (F *vs.* M)	67 *vs.* 52	1.54 (1.08-2.2)	0.05	0.062	-	-	-
	Transcript type e14a2 e13a2 e14a2/e13a2	 59 55 67	 - 0.64 (0.43-1.44) 0.87 (0.53-1.45)	 - 0.34 0.60	 0.6	-	-	-
MR4.5 (N of events = 76/200)	No AGA *vs.* AGA	47 *vs.* 27	0.53 (0.31-0.94)	0.015	0.037	0.54 (0.32-0.82)	0.004	
	ELTS Low Intermediate High	 48 21 18	 - 0.29 (0.14-0.60) 0.32 (0.13-0.81)	 - <0.001 0.016	 0.005	 - 0.51 (0.32-0.80) 0.43 (0.24-0.75)	 - 0.004 0.003	 Wald test: *P*=0.019
	Age (<63 *vs.* ≥63 yrs)	35 *vs.* 52	1.54 (0.87-2.72)	0.13	0.163	-	-	-
	Sex (F *vs.* M)	44 *vs.* 33	0.64 (0.41-1.09)	0.055	0.091	-	-	-
	Transcript type e14a2 e13a2 e14a2/e13a2	 43 33 42	 - 0.68 (0.41-1.13) 0.90 (0.64-1.64)	 - 0.13 0.74	 0.46	-	-	-

Variables with *P*≤0.05 in univariate analysis following false-discovery correction were included in the multivariable analysis models. CI: Confidence Interval; N: number; AGA: additional genetic abnormalities; FDR: false discovery rate; F: female; M: male; KD: kinase domain; MMR: major molecular response; yrs: years; ELTS: EUTOS long-term survival score. For ELTS clinical risk groups, *P* values are compared with the low-risk group. *Univariate and multivariable analysis for kinase domain mutation acquisition, MMR, MR4 and MR4.5 were assessed with Fine and Gray modeling. This utilizes sub-distribution hazard ratios, which are reported here.

sequencing was performed.[17,27] The *ASXL1* subclone became undetectable, whereas the second 3-mutant subclone expanded and evolved at BP. Eight additional variants were detected at BP, including six *ABL1* kinase domain mutations. In contrast, patient 384 had a single *ASXL1* nonsense variant at diagnosis and an excellent response to imatinib therapy. A deep molecular response was achieved at six months and the *ASXL1* variant became undetectable by four months. The epigenetic modifier *ASXL1* may not harbor intrinsic transformation potential, and additional genomic events may be necessary for progression.

Discussion

This study, involving a large unselected cohort of consecutively treated CP-CML patients initiated with first-line imatinib,[27] expands the results of our prior genomic analysis of a highly selected patient cohort.[17] A higher frequency of AGA at diagnosis was an independent predictor of treatment failure and inferior molecular responses. The data also expand the findings of other genomic studies where patients were retrospectively selected for sequencing based on their known clinical outcome.[22,23] Whether more potent TKI when administered as first-line therapy can abrogate the inferior responses associated with AGA[22] needs to be further investigated. However, the results presented in this study suggest a highly proactive approach with TKI switch for failure and sub-optimal molecular response may not completely negate the adverse impact of AGA. In our unselected CML cohort, AGA were detected in 31% of patients at diagnosis, and were associated with inferior FFS and molecular responses, even when patients were treated with a highly proactive intervention strategy involving imatinib dose escalation and/or switch to nilotinib for suboptimal molecular responses. For example, the 4-year rate of MR4 in ENESTnd was 32% in the imatinib-treated cohort

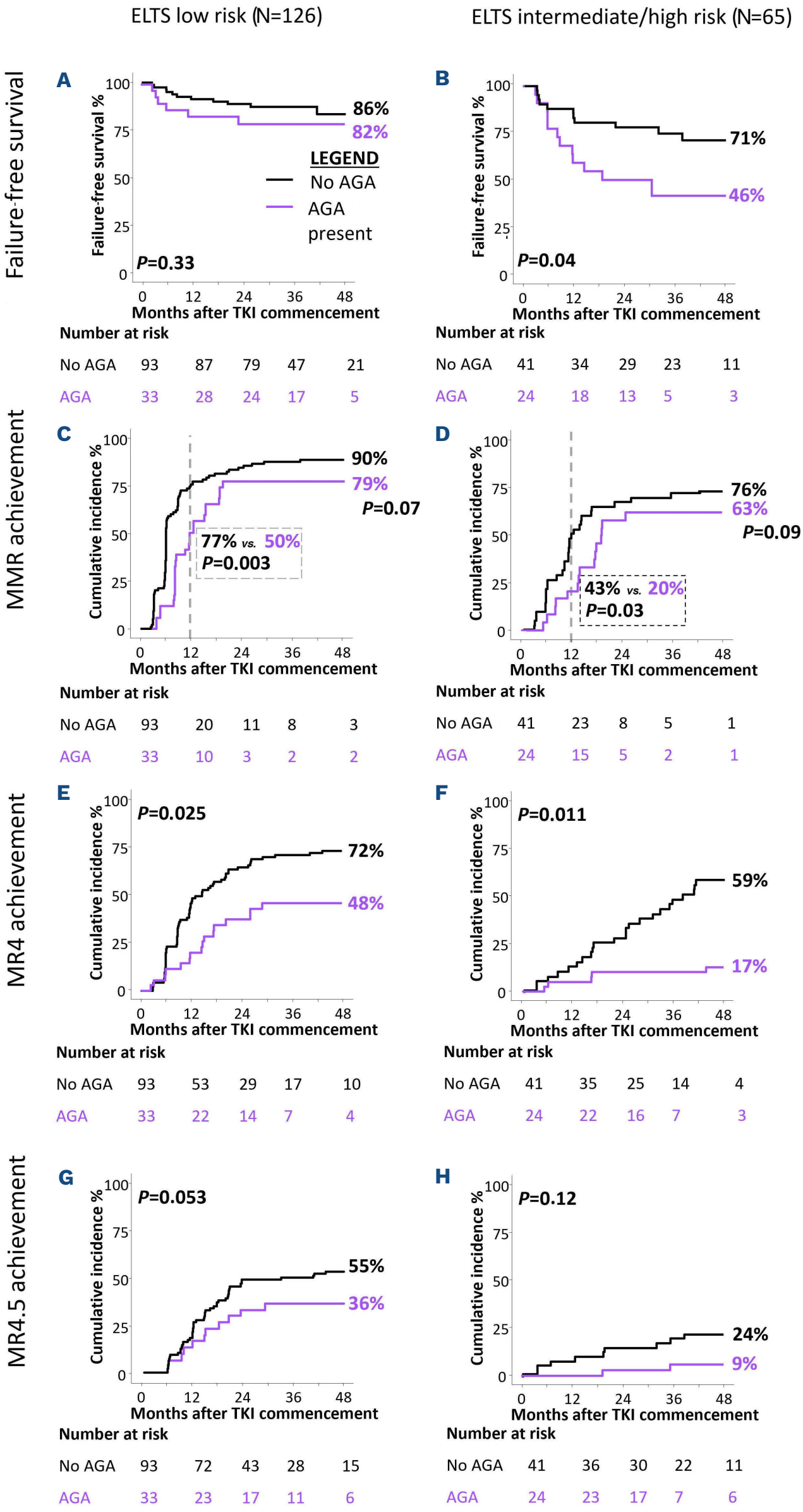

Figure 4. Failure-free survival and molecular response among the EUTOS long-term survival score risk groups according to additional genetic abnormalities at diagnosis. Failure-free survival (FFS) according to (A) low and (B) intermediate-/high-risk EUTOS long-term survival score (ELTS). The 48-month cumulative incidence of major molecular response (MMR) according to (C) low and (D) intermediate-/high-risk ELTS; 12-month values indicated. The 4-year cumulative incidence of MR4 according to (E) low and (F) intermediate-/high-risk ELTS, and the 4-year cumulative incidence of MR4.5 according to (G) low and (H) intermediate-/high-risk ELTS. TKI: tyrosine kinase inhibitor.

where patients were randomized to receive 400 mg daily of imatinib.[3] In comparison, the 4-year rate of MR4 in the TIDEL-II study population was 59%, which is similar to the MR4 rate achieved by the matched timepoint in the nilotinib 300 mg twice daily arm of the ENESTnd study (56%).[3] Therefore, conventional dosing of imatinib (i.e., 400 mg daily) will likely produce more marked differences than observed in this study.

The independent predictors of patient outcome (inferior FFS, acquisition of *BCR::ABL1* kinase domain mutations, lower rates of MMR, MR4 and MR4.5) were AGA at diagnosis and the ELTS clinical risk score. No variable predicted OS or progression to AP/BP, but progression was uncommon in the study population. Combining AGA and the ELTS risk category further differentiated subgroups with inferior molecular responses. In particular, patients with intermediate-/ high-risk ELTS who had AGA at diagnosis had inferior FFS and molecular responses. Notably, none of the eight patients in the high-risk ELTS group with AGA achieved MMR by 12 months or MR4 and MR4.5 by four years.

ASXL1 variants have been associated with an inferior outcome in many myeloid malignancies, including CML.[31] Furthermore, it is among the most frequently mutated genes at BP of CML.[17,23,32,33] Mutated *ASXL1* was associated with lower response rates to the third-generation TKI, olverembatinib, and higher risk of progression and death.[31] Consistent with other studies,[33] *ASXL1* was the most frequently mutated cancer gene at diagnosis in our cohort (9%), and *ASXL1* variants were associated with inferior FFS and higher risk of *BCR::ABL1* kinase domain mutation development compared with other AGA. The most frequently reported *ASXL1* variant in myeloid neoplasms is G646Wfs*12.[34] The reliable detection of this mutant above background reads using next-generation sequencing requires a simple set of metrics.[35] Only two patients in our cohort (1%) had the ASXL1 variant G646Wfs*12 at the time of diagnosis. In contrast, this variant has recently been reported at a high frequency in CP- or AP-CML patients with resistance to imatinib and/or second-generation TKI.[31] The frequency of specific *ASXL1* variants may vary depending on the disease phase, and characterization of much larger cohorts will be needed to confirm the prognostic effect of variants in individual genes. Ochi *et al.* assessed clonal evolution of genetic abnormalities in blastic transformation of CML.[32]

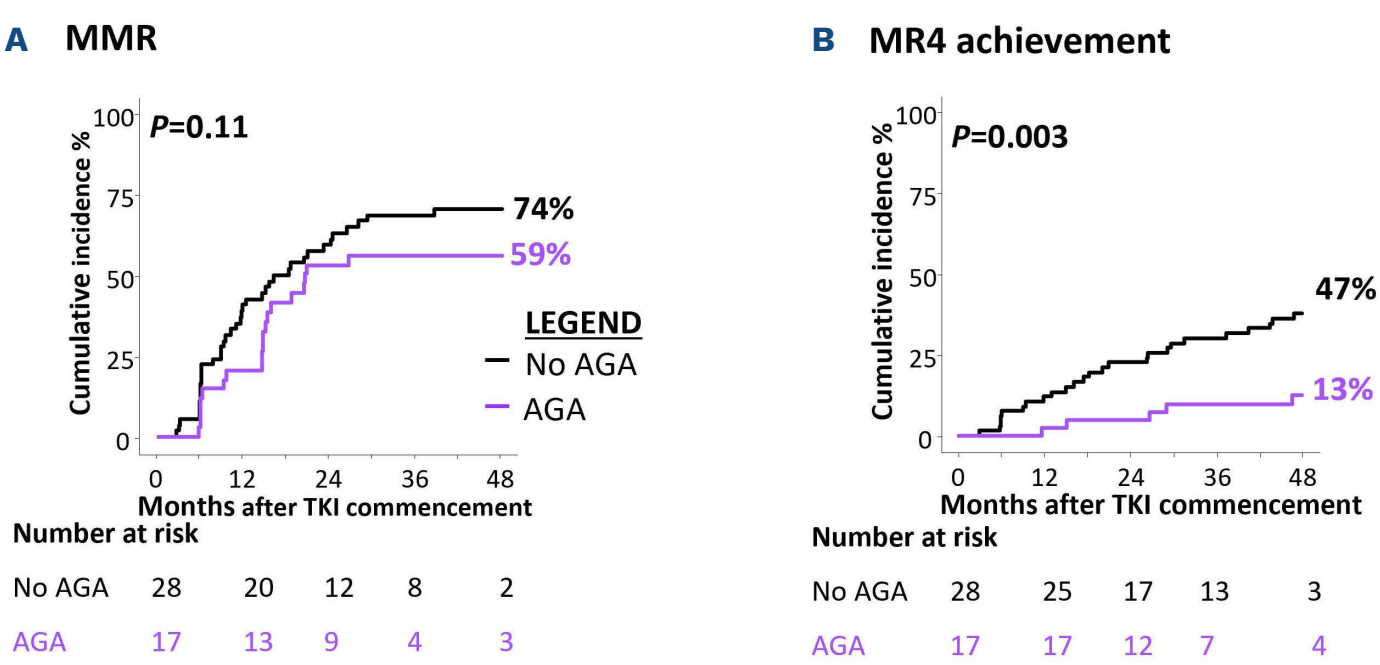

Figure 5. Impact of additional genetic abnormalities on molecular response for patients who switched to nilotinib for trial-defined failure criteria. Outcomes and responses according to additional genetic abnormalities (AGA) status at diagnosis in patients who switched to nilotinib for trial-defined nilotinib switch criteria, excluding imatinib intolerance. (A) Cumulative incidence of major molecular response (MMR). (B) Cumulative incidence of MR4 by 4 years. (C) Cumulative incidence of MR4.5 by 4 years. TKI: tyrosine kinase inhibitor.

Almost all patients with mutant *ASXL1* gained additional genetic abnormalities during progression to BP, suggesting that mutant *ASXL1* clones may gain a more transforming phenotype through clonal evolution. Targeted therapeutic approaches for mutant *ASXL1* could be a possible future treatment strategy. Mutant *ASXL1* was reported to drive response to the *BCL2* inhibitor venetoclax *in vitro*[36] and a small molecule inhibitor of *BAP1* inhibited mutant *ASXL1*-driven leukemic gene expression and impaired tumor growth *in vivo*.[37]

Ph-associated rearrangements are a novel genomic level event associated with the formation of the Ph chromosome that has only recently been described in CML.[17] We found a strong correlation between the presence of Ph-associated rearrangements and the acquisition of *BCR::ABL1* kinase domain mutations and significantly inferior molecular responses. Every rearrangement was novel and unique to individual patients. Whether these structural rearrangements directly influence response and outcome or if they are a marker of genomic instability is unknown. Ph-associated rearrangements may be associated with defective DNA double-strand break repair. When DNA repair mechanisms are not properly regulated, genome integrity may not be maintained. Major repair mechanisms of double strand breaks are homologous recombination or the error prone non-homologous end joining. It is not yet known whether homologous recombination repair deficiency plays a role in the generation of Ph-associated rearrangements, which could trigger use of error prone non-homologous end joining repair.[38-41] Complexity associated with the formation of the Ph chromosome for some patients was revealed by using higher resolution next-generation sequencing techniques, demonstrating added layers of genomic intricacies beyond the previously reported derivative 9 deletions. Furthermore, Ph-associated rearrangements were strongly associated with the absence of the *ABL1::BCR* reciprocal transcript, which is consistent with disruption of sequences adjacent to breakpoints on the derivative chromosomes. It is also consistent with the absence of *ABL1::BCR* transcripts previously reported for patients with large deletions adjacent to the translocation breakpoints identified by fluorescence *in situ* hybridization.[42,43] Some rearrangements involved genomic inversions, fragmentation, circularization and random reassembly, potentially from genomic 'shattering'. These have been described in other malignancies and associated with therapy resistance.[44] The Ph-associated rearrangements emphasize that the formation of the *BCR::ABL1* gene fusion in some patients may result from a multi-step process.[45,46] Similar rearrangements have been identified in other fusion-based hematologic malignancies, such as acute promyelocytic leukemia,[47,48] characterized by the *PML::RARA* fusion, and *RUNX1::RUNXT1*-mutated acute myeloid leukemia,[49,50] a subtype generally associated with a favorable prognosis. In both contexts, these genomic findings have been associated with poor outcomes.[47,49] The promiscuous *KMT2A* gene defines a category of aggressive high-risk leukemia with over 100 fusion partners.[51,52] A subset of these patients had complex chromosomal rearrangements, internal tandem duplications, focal gene deletions, and 11q chromosomal inversions and insertions.[51] While further investigation is required to clarify the clinical and biological significance of

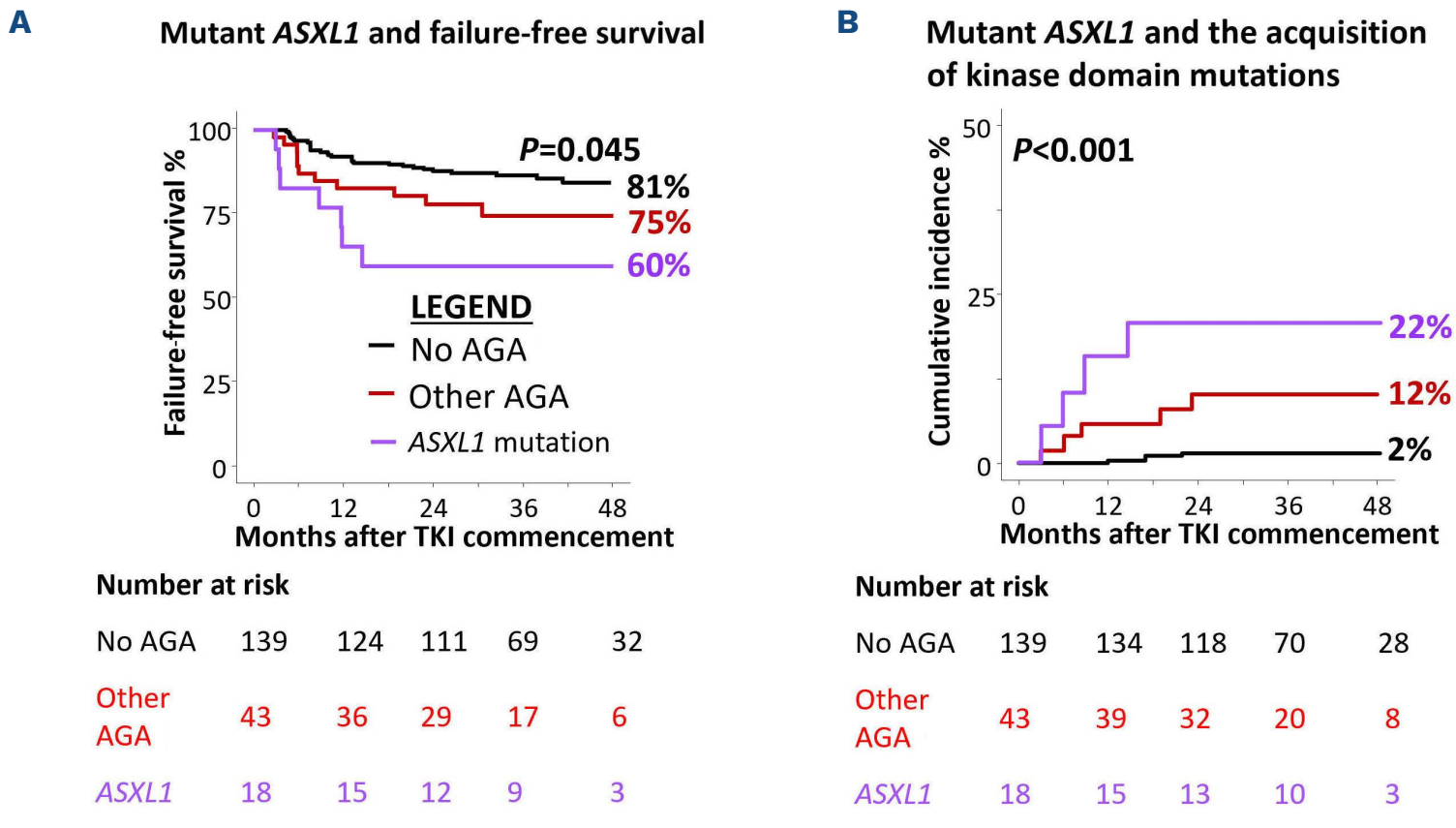

Figure 6. *ASXL1* variants were associated with treatment failure, including the acquisition of *BCR::ABL1* kinase domain mutations. *ASXL1* variants predicted for (A) inferior failure-free survival (FFS) and (B) the acquisition of *BCR::ABL1* mutations compared with other additional genetic abnormalities (AGA) or no AGA at diagnosis.

these rearrangements, they may be a hallmark of genomic instability. A customized RNA-capture panel, designed specifically to identify novel genomic rearrangements associated with the expected fusions characterizing the specific leukemias, could illuminate the complexity of these events. Risk stratification in CML in the TKI era has relied upon clinical and laboratory parameters within the Sokal risk score, and more recently has included the ELTS score. The rate of early *BCR::ABL1* decline in response to TKI therapy has also been utilized to differentiate patient outcomes, with TKI switch recommended for patients failing to adequately meet the critical molecular milestones recommended within the first 12 months of TKI initiation. However, there is increasing awareness of the influence of cancer-gene variants for risk stratification in other hematologic malignancies, and genetic profiling has been incorporated into diagnostics, prognostication and treatment algorithms.[53-56] Our work supports and expands the landscape of AGA and reveals their effect on outcomes for patients with CML. Exploration of the complexities of Ph-associated rearrangements will also be useful as a novel predictor of adverse outcomes in CML, and may pave the way for similar findings in other fusion-based cancers. While the TIDEL-II therapeutic approach does not reflect current treatment practices (where patients are treated with first-line second-generation TKI or imatinib at a standard dose of 400 mg), it highlights the fact that, despite higher dose first-line imatinib, aggressive monitoring and proactive TKI switching, the negative effect of AGA could not be overcome. Whether upfront treatment with more potent second-generation TKI can nullify the inferior outcomes we observed for patients with AGA at diagnosis remains to be established. If this is proven, optimal TKI selection in CML could integrate the genomic landscape into risk stratification, in addition to other factors that influence selection of therapy, such as toxicity profile and patient comorbidity. Early consideration of allogeneic stem cell transplantation or novel therapeutic approaches in patients stratified as very high risk of treatment failure and inferior molecular responses based on their individual genetic and clinical profile may be justified. Very high-risk features may include the detection of multiple AGA at diagnosis, as was observed for patient 430 who had rapid progression at four months of imatinib (Figure 2). Expanded data are required to establish the genomic criteria that define very high risk.

Disclosures

NS received honoraria from Novartis and meeting sponsorship from Novartis, Amgen, and Janssen. SB is a member of the advisory boards of Qiagen, Novartis and Cepheid, and received honoraria from Qiagen, Novartis, Bristol-Myers Squibb, Incyte and Cepheid, and research funding from Novartis and Cepheid. ASY is a member of the advisory board for Novartis, and received research funding from Novartis, Bristol-Myers Squibb and Celgene, and honoraria from Novartis and Bristol-Myers Squibb. AKM is a member of the advisory boards of Sobi and Novartis, and received speaker fees from Abbvie and meeting sponsorship from MSD and Amgen. DMR has received research funding and honoraria from Novartis and Bristol-Myers Squibb and honoraria from Takeda. APG received honoraria from Roche, MSD, Janssen, Novartis and Amgen while having advisory roles for MSD Oncology, Janssen and Novartis. TPH is a member of the advisory boards and has received research funding and honoraria from Novartis and Bristol-Myers Squibb. The other authors declare no conflicts of interest.

Contributions

NS collected and analyzed the data, and wrote the paper. CW designed the probes for the RNA capture panel, prepared samples, and analyzed the data. NHS, VS, RRK, ML, JB and HA prepared samples and completed the required laboratory work. AWS, JF, PW, DT and CHK performed the bioinformatic analysis and pipeline development. IP, ASY, RMK, DMR, AKM, APS, APG, DTY and HS reviewed the manuscript. DTY, APG and TPH designed and conducted the TIDEL II trial and co-ordinated the correlative studies. TPH contributed key concepts and assisted in writing the paper. SB designed the research, analyzed the data, contributed key concepts and methodology, and assisted in writing the manuscript.

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Data-sharing statement

De-identified participant data collected for our study can be made available to researchers once appropriate ethical approval and a signed data access agreement is obtained.

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