

Progression in patients with low- and intermediate1-risk del(5q) myelodysplastic syndromes is predicted by a limited subset of mutations

Christian Scharenberg^{1,2}, Valentina Giai¹, Andrea Pellagatti³, Leonie Saft⁴, Marios Dimitriou¹, Monika Jansson¹, Martin Jädersten¹, Alf Grandien¹, Iyadh Douagi¹, Donna S. Neuberg⁵, Katarina LeBlanc¹, Jacqueline Boulton³, Mohsen Karimi¹, Sten Eirik W. Jacobsen^{1,6}, Petter S. Woll¹, and Eva Hellström-Lindberg¹

1 Department of Medicine, Center for Hematology and Regenerative Medicine, Karolinska University Hospital Huddinge, Karolinska Institutet, Stockholm, Sweden [✉](#)

2 Department of Medicine, Division of Hematology, Skaraborgs Hospital Skövde, Sweden [✉](#)

3 Bloodwise Molecular Haematology Unit, Nuffield Division of Clinical Laboratory Sciences, Radcliffe Department of Medicine, University of Oxford, and NIHR Biomedical Research Centre, Oxford, UK [✉](#)

4 Department of Pathology, Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden [✉](#)

5 Department of Biostatistics and Computational Biology, Dana–Farber Cancer Institute, Boston, USA [✉](#)

6 Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden

[✉](#)

Corresponding author

Eva Hellström-Lindberg, M.D. PhD,[✉](#)
Karolinska Institutet,[✉](#)Department of Medicine,[✉](#)
Center for Hematology and Regenerative Medicine,
Karolinska University Hospital, Huddinge,
SE-141 86 Stockholm, Sweden,
Phone: +46-8-585 800 00
email: eva.hellstrom-lindberg@ki.se

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Author Contributions

CS, VG, AP, LS, ID, MJ and MK conducted experiments. PSW, MJ, AG, DN, KLB, JB, SEJ and EHL advised on experiments. CS, PSW, VG, AP, MD, MJ, DN, MK analyzed the data. CS and EHL

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Abstract

A high proportion of patients with lower-risk del(5q) myelodysplastic syndromes (MDS) will respond to treatment with lenalidomide. Median duration of transfusion-independence is 2 years with some long-lasting responses, but almost 40% of patients progress to acute leukemia by 5 years after start of treatment. Mechanisms underlying disease progression other than the well-established finding of small TP53-mutated subclones at diagnosis remain unclear. We studied a longitudinal cohort of 35 low- and intermediate-1-risk del(5q) patients treated with lenalidomide (n=22) or not (n=13) by flow cytometric surveillance of hematopoietic stem and progenitor cells (HSPC) subsets, targeted sequencing of mutational patterns, and changes in the bone marrow microenvironment. All 13 patients with disease progression were identified by a limited number of mutations in TP53, RUNX1, and TET2, respectively, with PTPN11 and SF3B1 occurring in one patient each. TP53 mutations were found in 7 of 9 patients who developed acute leukemia, and were documented to be present in the earliest sample (n=1) and acquired during lenalidomide treatment (n=6). By contrast, analysis of the microenvironment, and of HSPC by flow cytometry was of limited prognostic value. Based on our data, we advocate conducting a prospective study aimed at investigating, in a larger number of del(5q) MDS cases pre- and post-lenalidomide treatment, whether the detection of such mutations can guide clinical decision making.

Introduction

One salient feature of malignant hematopoiesis is clonal dominance, i.e., the suppression of normal hematopoiesis by the neoplastic clone. In myelodysplastic syndromes (MDS) associated with deletion of the long arm of chromosome 5 (del(5q)), clonal dominance leads to the expansion of del(5q) hematopoietic stem cells (HSC) at the expense of normal HSC ¹. In a recent study we demonstrated that rare HSC carrying del(5q) are necessary and sufficient to propagate the disease ². Furthermore, we found del(5q) HSC to be selectively resistant to lenalidomide at the time of complete clinical and cytogenetic remission ³, potentially enabling the continual accrual of mutations and disease progression. At diagnosis, the majority of MDS patients carry recurrent mutations in a number of myeloid candidate genes, several of which are strongly associated with outcome ⁴⁻⁷. We observed this pattern also in del(5q) MDS, where 64% had additional mutations detected in the HSC compartment. However, del(5q) seemed to precede the identified driver mutations in most cases, arguing that deletion of 5q is sufficient for a clonal advantage ².

The immunomodulatory drug lenalidomide has a specific effect in patients with lower-risk del(5q) MDS, abrogating the need for transfusions in around 50% of patients ^{8,9}. The corresponding incidence of complete cytogenetic remissions (CCyR) varies between 16 and 26% ⁹. While a small subgroup of patients may maintain complete remissions for years even after the withdrawal of lenalidomide ¹⁰, the median response duration is two years ⁹ and approximately 40% of the patients in the MDS 004 study had progressed to acute myeloid leukemia (AML) at 5 years ¹¹.

As the molecular mechanisms underlying disease progression in del(5q) MDS remain to be elucidated, we do not know how to predict disease progression or how to monitor patients during lenalidomide treatment. We previously reported that small TP53 mutated subclones predict for an unfavorable outcome in del(5q) patients, and that these subclones expand with disease progression ¹². However, whether or not other somatic mutations or factors related to the bone marrow microenvironment also contribute to disease progression has not been comprehensively assessed. In this longitudinal study, we show for the first time that all patients with disease progression were identified by a limited subset of mutations. Based on our data, we therefore advocate that mutational profiling should be used before and during treatment of del(5q) MDS patients in order to guide individual clinical decisions.

Methods

Clinical characteristics of patient cohort

Between 2004 and 2015 we included consecutive patients at the Karolinska University Hospital in Stockholm, Sweden based on the following criteria: MDS with IPSS low or intermediate-1 risk at diagnosis and standard cytogenetics including del5q31 without or with one additional abnormality. All patients were followed until April 2016 for survival, disease progression, and treatment. In total, 35 patients were analyzed by targeted sequencing for frequently mutated genes with known or putative implications in the pathogenesis of myeloid diseases, and material from the vast majority of patients was available for flow cytometric analysis of hematopoietic stem- and progenitor cells (HSPC). In six patients we were able to FACS-purify HSPC from two or more consecutive visits for further studies. The above studies of MDS patients, as well as a study of healthy individuals were approved by the institutional review board at Karolinska Institute and both patients and healthy individuals provided written informed consent. As a definition for clinical progression, we used the same definition as in Jadersten et al.¹². Besides defining “progression” as patients who developed AML (n=9 cases), 4 cases were defined as progression based on acquisition of additional karyotypic abnormalities (n=3) and increase of marrow blasts from <5 to 11% (n=1) in combination with worsening cytopenias.

Flow Cytometry, Cell Sorting and analysis of gene expression

Hematopoietic stem cells (HSC), Multipotent Progenitors (MPP), Lymphoid-primed multipotent progenitors (LMPP) and three subsets of myeloid progenitors, including common myeloid progenitors (CMP), granulocyte–macrophage progenitors (GMP), and megakaryocyte–erythroid progenitors (MEP) were identified using a panel of antibodies based on the following surface markers^{13,14}: HSC (lin- CD34+ CD38- CD90+ CD45RA-), MPP (lin- CD34+ CD38- CD90- CD45RA-), LMPP (lin- CD34+ CD38- CD90- CD45RA+), CMP (lin- CD34+ CD38+ CD123+ CD45RA-), GMP (lin- CD34+ CD38+ CD123+ CD45RA+); MEP (lin- CD34+ CD38+ CD123- CD45RA-). Cell populations were isolated from CD34-enriched normal and MDS mononuclear cells by fluorescence-activated cell sorting (FACS) on a FACS Aria and used for subsequent analyses. Gene expression was analyzed by Fluidigm Dynamic Arrays as previously described³ (Supplementary Figure 5).

Fluorescence in situ hybridization

Flow sorted cell populations were spun onto glass slides. Slides were subsequently treated with pepsin and fixed with formaldehyde/MgCl₂. To detect deletions of 5q31, the LSI EGR1/D5S721, D5S23 Dual Color Probe (Abbott-Vysis, Downers Grove, IL) was used; LSI

EGR1 detects deletions of 5q31, and LSI D5S721, D5S23 detects 5p15.2 and serves as an internal control. Probes were applied as recommended by the manufacturer. As FISH analysis does not detect additional cytogenetic changes, standard cytogenetics was performed on mononuclear cells from the same time points.

Bone marrow morphology and IHC

Sequential bone marrow samples were assessed by routine morphology and immunohistochemistry at each time point. Bone marrow samples were assessed at diagnosis in all patients while five were analyzed prior to and at various time points during treatment with lenalidomide (MDS063, 094, 106, 110, and 143). BM cellularity and fibrosis were assessed according to European consensus guidelines¹⁵. Immunohistochemistry was performed for different markers including p53 DO-1 (Santa Cruz, Biotechnology, Inc)¹¹, CD34, CD68, Nestin, CD271, CD146 (Novocastra, UK), using the automated BondTM and Ventana Bench Mark XT systems according to the manufacturers' instructions. The microvascular density (MVD) was quantified as the number of blood vessels per high power field (HPF), using regular light microscopy at high (400x) magnification as previously described¹⁶. Blood vessels were identified as CD34 positive endothelial cells forming a structure with a clearly discernable lumen. The frequency of CD34+ mononuclear cells and the tendency of CD34+ cells to form clusters were assessed as previously described¹⁷.

MSC cultures & RNA isolation

Mesenchymal stromal cells were isolated from six untreated del(5q) cases and six healthy volunteers using a previously published standard procedure¹⁸ and expanded as previously detailed while fulfilling uniformly the minimal MSC criteria¹⁹. Cell lysates were harvested with lysis buffer (Qiagen, Hilden, Germany), RNA was extracted using a Qiagen RNeasy minikit, and then stored in RNase-free water at -80 C.

Affymetrix gene expression of MSC

Gene expression profiling of MSC was performed as was previously described for CD34+ cells^{20,21}. Briefly, for each sample 100 ng of total RNA were amplified and labelled with the 3' IVT Express Kit (Affymetrix, Santa Clara, CA) following the manufacturer's recommendations. Biotin-labelled fragmented cRNA was hybridized to GeneChip Human Genome U133 Plus2.0 arrays (Affymetrix), covering over 47,000 transcripts. Hybridization was performed at 45°C for 16h in Hybridization Oven 640 (Affymetrix). Chips were washed and stained in a Fluidics Station 450 (Affymetrix) and scanned using a GeneChip Scanner 3000 (Affymetrix). Affymetrix CEL files were pre-processed using the robust multiarray average (RMA) algorithm²². Data analysis was performed using GeneSpring 12.6 (Agilent Technologies).

Quality control results obtained for scale factors, background levels, percentage of present calls, 32/52 GAPDH ratio, and intensities of spike hybridization controls were within the acceptable range for all samples²³.

DNA sequencing & bioinformatics analyses

Haloplex target enrichment for Illumina (Agilent) was applied for mutation screening in panels of either 42 or 74 frequently mutated genes (supplementary Table 1) according to manufacturers instruction. Of note, the 42-gene panel covers most genes reported to be recurrently mutated in MDS⁴⁻⁶ and were included in both kits. Briefly, bone marrow mononuclear cells were separated by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). Genomic DNA was extracted using GeneElute DNA extraction kit (Sigma) and quantified by Qubit. All samples were individually barcoded using 96 barcoding oligos by Agilent during enrichment and quality of individual libraries were checked by Tape Station D1K assays (Agilent). Sequencing was performed on pooled samples either using HiSeq 2000 (Illumina) sequencer through paired-end, 100 bp reads or MiSeq sequencer through paired-end, 150 bp reads. Illumina Sequencing adapters were removed using Cutadapt (v.0.9.5) and reads were aligned to the hg19 using MosaikAligner (v.2.1.33). Sequence variant were identified using VarScan 2 in mpileup2cns mode. Variants were annotated using ANNOVAR²⁴. ECD DNA control included in Haloplex kit was used to filter out sequencing errors. Variants were selected for further analysis if they meet the following criteria: 1) minimum coverage of 100X, 2) minimum of 20 variant reads, 3) having a variant allele frequency of >0.03 for all genes except for *TP53* where the limit of detection was set ≥ 0.01 based on our previous studies that demonstrated these detection levels to be clinically relevant^{11,12,25}, 4) not present in 1000 Genome database, 5) not listed in dbSNP unless listed in COSMIC 65 database, 6) truncating or damaging based of SIFT if not present in COSMIC 65. Sequencing results for case MDS019 were obtained by exome capture performed using SureSelect Human All Exon 50Mb (Agilent, Santa Clara, CA). Sequencing was done on the Illumina Genome Analyzer IIx platform. Sequencing results for case MDS110 were previously published².

Statistical Analysis

Survival and time to progression (defined as blast increase >10% or acquisition of complex karyotype) were updated April 2016 and were measured from time of diagnosis. Continuous variables were compared using Mann-Whitney U-test or Student's t tests, as appropriate. Categorical variables were analyzed by Fisher's exact test. All statistical calculations were performed using Graph Pad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Patient outcome.

We analyzed 35 IPSSS low and INT-1 risk patients with del(5q) at one or more time points by targeted sequencing (Supplementary Tables 1 and 2). Patients were allocated to receive lenalidomide treatment (n=22) or not (n=13) based on severity of anemia, co-morbidities and availability of other therapeutic options, such as allogeneic stem cell transplantation. After a median observation time of 61 months (range 0.5-187) from diagnosis, 17 patients remained alive. The median survival of all patients (median age 82, range 45-96) was 102 months (range 0.5-187). Amongst these were six patients who underwent allogeneic stem cell transplantation at 145, 135, 116, 42, 12 and 6 months post-diagnosis, respectively, with 5 of 6 SCT-patients still surviving (median age at transplantation 63 years, range 46-73). The median survival for the 29 patients who did not undergo transplantation was 70 months. Further demographic and clinical characteristics of the patients are detailed in Supplementary Figure 1.

Disease progression is associated with the emergence of new mutations

In total, 84% of patients had a recurrent mutation in at least one gene in our panels. The summarized results of the targeted sequencing are shown in supplementary Table 2. We found no differences in other clinical parameters (e.g., age, blood counts, or additional cytogenetic abnormalities) between patients in whom we found recurrent mutations and those in whom we did not. Considering all time points within a patient, the most frequently mutated genes were *TP53* (n=11 patients), *DNMT3A* (n=8), *TET2* (n=7), *ASXL1* (n=6) and *RUNX1* (n=3) (Figure 1B). Interestingly, the mutational landscape seemed to differ from that of lower-risk MDS in general, as described in earlier reports: while mutations in genes involved in splicing were less frequent, the spectrum of mutations in this pure del(5q) cohort was more similar to that of high-risk MDS^{4-6,26}.

Diagnostic or pre-treatment samples were available for 14 of 22 patients treated with lenalidomide (the LEN cohort), and all (13/13) patients who did not receive lenalidomide (the 'no LEN' cohort), and there were no significant differences in the number or type of mutations between these 2 groups (p>0.99). Only 2 out of 22 patients failed to respond to lenalidomide treatment and although both patients harbored mutations, meaningful statistical analysis of these two patients was not possible.

Of the 35 patients, thirteen (37%) progressed to high-risk MDS (RAEB-1, n=3 and RAEB-2, n=1) or leukemia (n=9) after a median of 85 months (range, 31-184) post diagnosis. Of the 27 patients in whom diagnostic or pre-treatment samples were available, nine (33%) patients showed no mutations, while 18 (67%) had one or more mutation (supplementary Table 1). The presence of any recurrent mutation covered by these MDS panels early in their disease-course and prior to treatment did not predict progression (p=0.68). However, when considering the 20 patients whose samples were neither from diagnosis nor pre-treatment, absence of mutations was suggestive of freedom from progression (p=0.073).

In sixteen patients, material was available from more than one time-point, enabling longitudinal assessment of allelic burden in relation to treatment with lenalidomide or SCT. Progression was associated with the detection of a restricted subset of new recurrent mutations, either alone or in combination (Figure 1B): *TP53* (n=9, p=0.0004), *TET2* (n=6, p=0.006), *RUNX1* (n=3, p=0.044). In addition, we observed mutations in *SF3B1* and *PTPN11* in two single cases. Longitudinal samples were available for all nine patients with leukemic transformation (MDS019, 038, 063, 075, 106, 110, 143, 155, 175). Interestingly, we detected *TP53* mutations in seven of these nine patients (MDS038, 063, 075, 106, 143, 155, 175) (Figure 2A, C-F), confidently detected already in the earliest sample in only 1 case (MDS038) and acquired in 6 cases (MDS063, 075, 106, 143, 155, 175). Overall, there was a high correlation between the detection of a *TP53* mutation by targeted sequencing and cells staining strongly positive for *TP53* by immunohistochemistry (Supplementary Table 4). Importantly, of 6 patients with evidence of acquisition of mutations in *TP53* by targeted sequencing, 5 had been analyzed by deep sequencing without evidence of mutation at the diagnostic time point¹². Of these, 4 were negative for *TP53* by IHC. Interestingly, however, the fifth patient who was negative by deep sequencing indeed showed a positive IHC staining of 4 %.

In the remaining two patients with leukemic transformation, we detected mutations in *RUNX1* (n=1, MDS019, Figure 2B) and *TET2* (n=1, MDS110), present at both time points sampled from these patients. Patient MDS110 had also acquired a *NRAS* mutation at the later time point. Three patients transformed to higher-risk MDS and all carried mutations in *TET2* (MDS094, 096, 107). The only patient who progressed to RAEB-2 in the 'no LEN' cohort showed mutations in *TP53* and *EZH2*.

Regardless of whether the three mutations (*TP53*, *TET2* and *RUNX1*) were present in the initial sample or whether they subsequently developed, testing positive for any of them carried a high probability (13/16, 81%) for predicting progression. Follow-up time after the latest mutation screening was similar between patients who progressed (median of 18 months, range 1-91 months) and those who did not (median of 27 months, range 0.3-76 months).

In 11 out of 13 patients the new mutations were detected prior to the time point of clinical progression and the median time from detection of the mutation to clinical evidence of progression was 42 months (range 0-83.9). Thus, we were able to detect the mutation in the majority of cases well before clinical signs of disease progression (Figure 3).

Surveillance of HSPC subsets under lenalidomide therapy

In order to investigate the impact of lenalidomide therapy on distinct hematopoietic stem and progenitor cells in del(5q) MDS patients, we performed multicolor flow-cytometry. The distribution of sub-populations within the Lin-CD34+CD38- compartments, including HSCs, MPPs and LMPPs^{2,13} remained relatively unchanged in both diagnostic and LEN-treated del(5q) MDS patients compared to healthy controls (Figure 4). However, as previously reported^{2,27,28}, the GMP frequency within Lin-CD34+CD38+ cells was significantly suppressed in diagnostic del(5q) MDS with a concomitant increase in CMPs. Upon LEN treatment, the GMP and CMP distribution reverted to frequencies comparable to normal Lin-CD34+CD38+ cells.

Amongst the patients who progressed to leukemia serial samples were available for five patients including one patient who did not respond to LEN (MDS063). This allowed us to monitor kinetic changes in HSPC subsets within the same patient over time during treatment and disease progression (Figure 4C, supplementary Figure 2 and supplementary Table 3). While in each case there was one predominant HSPC subset that expanded prior to progression, the type of subset varied from patient to patient. We combined cell sorting with FISH analysis to assess the clonal size of distinct del(5q) HSPC subsets (Figure 4C and Supplementary Figure 3). Notably, although several of the patients investigated had a complete clinical response to lenalidomide, in all but one of these patients the mononuclear bone marrow cells and to a higher degree stem- and progenitor compartments contained a large fraction of 5q-deleted cells, and were thus not in complete cytogenetic remission.

Interestingly, in the only patient (MDS106) who initially showed a complete cytogenetic response based on FISH analysis of mononuclear bone marrow cells, also the myeloid progenitor subsets (CMP, GMP and MEP) showed minimal clonal involvement (Supplementary Figure 3B), whereas as much as 54% of the Lin-CD34+CD38-CD90+CD45RA- HSC compartment remained part of the del(5q) clone, supporting previous studies implicating a selective resistance of del(5q) HSCs to lenalidomide treatment.

The microenvironment in del(5q) and effects of lenalidomide treatment

To determine whether the failure to produce mature progeny is primarily intrinsic due to compromised stem- and progenitor cells or if extrinsic, micro-environmental factors contribute, we initiated MSC cultures from untreated del(5q) and healthy volunteers and generated gene expression profiles by Affymetrix microarray. While expression values for a variety of hematopoietic genes were minimal or absent, MSC cultures from both healthy volunteers and untreated del(5q) MDS patients expressed gene signatures typical for MSC (supplementary Figure 4). However, we found no statistically significant differentially expressed genes ($P < 0.05$, Welch t-test and Benjamini–Hochberg multiple testing correction), even when specifically looking for genes previously implicated in HSC-niche interactions²⁹ (Figure 5A).

We next investigated bone marrow biopsies in healthy controls and in del(5q) MDS patients before and during lenalidomide treatment. Material for longitudinal analysis by immunohistochemistry was available in five patients. These analyses revealed that microvessel density (MVD) was significantly higher in del(5q) MDS than in normal controls (MVD values of 5.2 ± 3.2 versus 2.4 ± 1.2 ; $p = 0.02$) but decreased during the initial phase of lenalidomide treatment in all five analyzed patients (Figure 5B). Subsequent therapeutic failure was associated with an increase in BM cellularity and MVD in 4 of 5 patients. The number of CD68+ macrophages was not increased in BM samples from del(5q) MDS patients as compared to controls, however, upon lenalidomide treatment a decrease was noted which was paralleled by a decrease in cellularity (Figure 5B). Surrogate markers for mesenchymal stem cells (e.g., nestin, CD271, CD146) demonstrated labeling restricted to perivascular mesenchymal cells including endothelial cells and adventitial sinusoidal cells.

Taken together, these experiments demonstrate that despite affecting microvessel density, lenalidomide did not exhibit its effects primarily via alteration of the cellular composition of the microenvironment based on the MSC markers tested.

Discussion

In this study we found that all patients with lower-risk MDS and isolated del(5q) who progressed to either higher-risk MDS or transformed to acute leukemia harbored recurrent mutations in *TP53*, *RUNX1*, and *TET2* in addition to the deletion 5q. Not surprisingly, we found that mutations increased in individual patients over time. While 62% of samples obtained before treatment showed mutations in addition to del(5q), 84% of samples carried mutations in the latest available time point, and several patients showed increased allele burdens and gain of new mutations during the course of disease and treatment. This suggests that clonal evolution is frequent in patients with lower-risk del(5q) MDS and argues that the del(5q) aberration is associated with marked clonal instability. By contrast, Chesnais et al. reported NGS data from 94 non-del(5q) lower-risk patients treated with lenalidomide ± EPO and found that only about one third of these patients had more than one genetic event, most often consisting of *SF3B1* plus one additional mutation. Moreover, response to lenalidomide was associated with a decrease in allelic burdens of the identified mutations, and only 2 of 18 patients analyzed at a later time point had acquired new recurrent mutations³⁰.

In our cohort, 13 of 35 patients progressed to either higher-risk MDS (n=4) or leukemia (n=9), 12 of whom were treated with lenalidomide. Seven of the nine patients who developed leukemia carried a *TP53* mutation. Based on a median sequencing depth of 370 reads, the mutation was considered present pre-treatment in one of these patients (MDS038) and to have developed under treatment in the other six. The presence of very small *TP53* mutated subclones pre-treatment cannot be excluded, but 5 of these 6 patients had previously been analyzed in a study by Jädersten et al. using deep-sequencing analysis (coverage of 1200X) and were found to be negative¹², and 4 also proved to be *TP53*-negative by IHC in the present study. Given that normal function of *TP53* is a requirement for apoptosis of erythroid cells due to haploinsufficiency of *RPS14*³¹, it is highly possible that *TP53* mutations may be selected out as a consequence of the deletion 5q. We observed that disease progression associated with the acquisition of *TP53* mutations was relatively common in these lenalidomide-treated del(5q) patients with some patients even exhibiting more than one *TP53* mutation. The marked clonal heterogeneity and instability revealed in this study is likely to play a role in disease progression of lower-risk del(5q) MDS treated with lenalidomide. While isolated del(5q) in lower-risk MDS has been associated with a relatively low risk for leukemic transformation compared to other MDS subtypes, del(5q) is known to

be associated with an adverse prognosis and a high incidence of *TP53* mutations in the context of complex karyotypes in newly diagnosed MDS as well as de novo AML³²⁻³⁴.

Our data show that although *TP53* was the most common molecular event at progression, the emergence of other mutations could be linked to either loss of treatment response or to progression. *RUNX1* mutations in our cohort were restricted to patients with disease progression and found in 3 of 13 patients. In none of these patients did the *RUNX1* allele burden suggest the presence of a germ-line mutation. *RUNX1* is a well established marker of poor prognosis in both MDS and AML³⁵⁻³⁷. Furthermore, we found mutations in *TET2* in 6 of 13 patients with evidence of disease progression. Although 3 patients had mutations in both *TP53* and *TET2*, our data do not provide evidence that this was not a result of independent mutational processes. We note that while mutations in *TET2* are relatively common in myeloid neoplasms in general^{38,39}, their impact in MDS is less clear^{40,41}, albeit one study reported that *TET2* mutations were associated with shorter survival in MDS patients undergoing HSCT⁴². Our data on del(5q) patients are in line with recent findings in myeloproliferative neoplasms where *TET2* mutations were associated with disease progression if they were acquired in a *JAK2* mutated subclone⁴³.

Our study of the clonal dynamics of all major HSPC *in vivo* shows that clonal advantage is not only a feature restricted only to MDS stem cells but also extends to the myeloid and erythroid progenitor compartments. Using flow cytometry for surveillance of HSPC subsets in lenalidomide-treated patients, we found that neither lenalidomide treatment nor the acquisition of additional mutations led to any uniform profound changes in the hematopoietic hierarchy unless the patient showed clinical signs of progression. Importantly, amongst patients who eventually progressed but initially had a complete clinical response, there was no difference between patients who reached a complete cytogenetic response and those who did not. Although lenalidomide temporarily reduced the size of the del(5q) stem and myeloid progenitor cell compartments, in no case did we observe complete clearance of del(5q) cells, and this was again irrespective of the mutational status of the patient.

Mutations in either tumor-suppressors or oncogenes have the potential to modify the competitive nature of cells, transforming them into either winners or losers with respect to normal cells⁴⁴. The relative cell fitness is dependent upon the cellular context and not simply

the result of altered cell proliferation. In this regard, the microenvironment is an important regulatory component when cancer cells compete with normal (stem) cells. However, our data do not support that the microenvironment in del(5q) MDS exerts a dominant constraint towards healthy hematopoiesis. Our studies of MSC grown *in vitro* confirm previous findings that the stromal component of the marrow microenvironment is not derived from the malignant clone in MDS⁴⁵. Microarray analysis exhibited an expression footprint consistent with MSC with high expression of MSC markers and absence of hematopoietic gene signatures. However, we observed only minor differences in gene expression between pre-treatment del(5q) and healthy mesenchymal stromal cells. While seemingly at odds with recent findings in cohorts of MDS with multiple subtypes^{46,47}, our studies in a pure del(5q) cohort are in line with earlier studies by other groups who found the stromal abnormalities to be reversible and that MDS stroma is able to support normal *in vitro* hematopoiesis^{48,49}.

In conclusion, while flow cytometric analysis of HSPC populations or analysis of the microenvironment had limited predictive value in this cohort of lower-risk del(5q) MDS, all patients who progressed to either higher-risk MDS or leukemia were identified by harboring recurrent mutations in a limited number of genes, i.e., *TP53*, *RUNX1*, and *TET2*. Based on our data, we advocate for conducting a prospective study aimed at investigating in a larger number of del(5q) MDS cases pre- and post-lenalidomide treatment, whether the detection of such mutations can guide clinical decision making, such as suggesting which patients should undergo hematopoietic cell transplantation.

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Figure legends

Figure 1. The mutational spectrum in del(5q) patients differs in untreated versus LEN-treated patients. (A) Study outline and clinical fate of patients untreated or treated with LEN. * denotes two patients who are alive and well after SCT. (B) Spectrum of mutations in relation to clinical outcome in LEN-treated versus untreated patients.

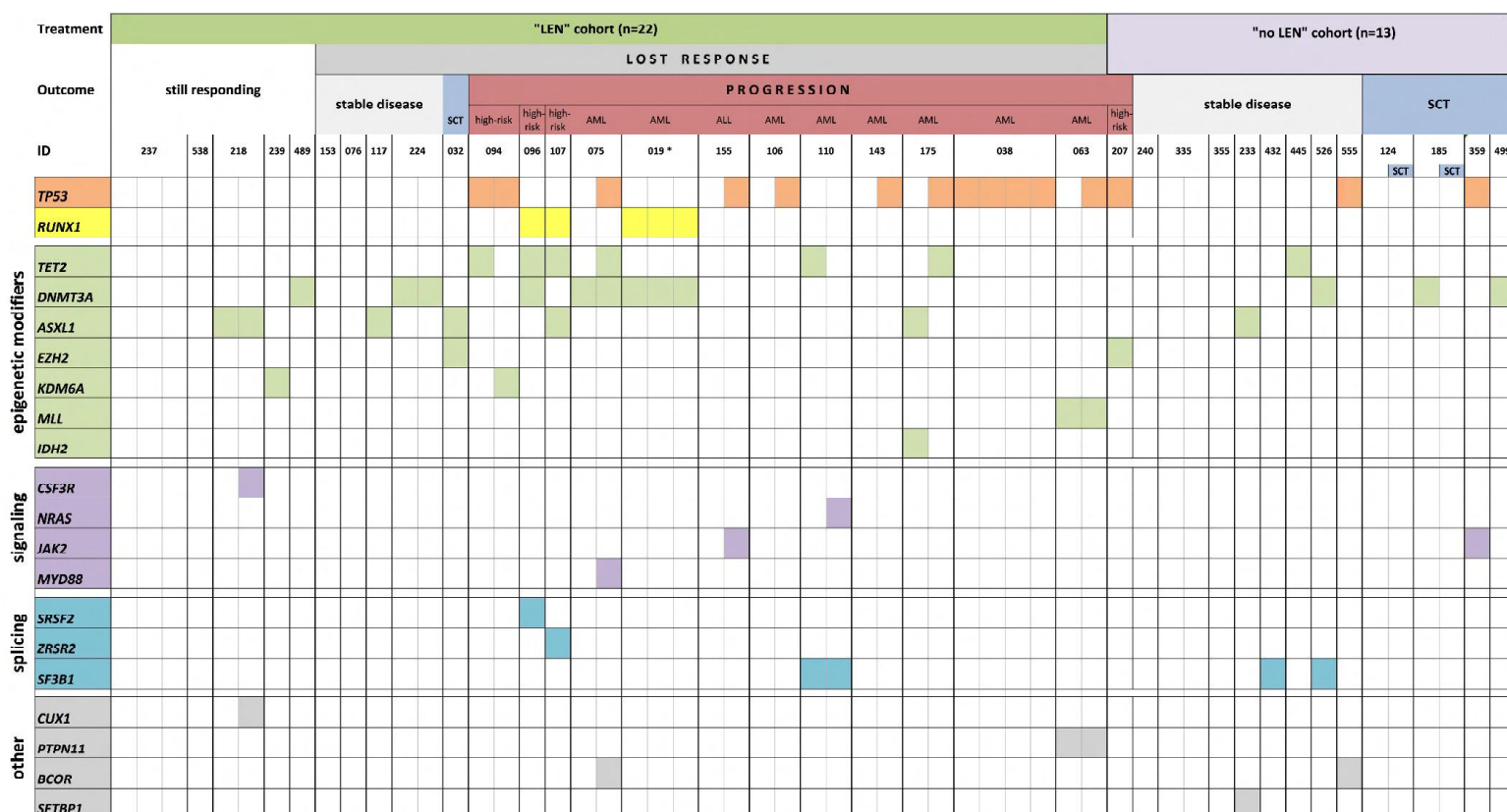
Figure 2. Longitudinal assessment of mutations during treatment with EPO (shaded in red) and LEN (shaded in grey). (A) Frequency of mutations in relation to the del(5q) clone in a patient who progressed to high-risk disease. (B) Variant allele frequency (VAF) in a patient who progressed to leukemia, received induction therapy and went into complete remission and was transplanted. *** This patient had trisomy 21, the region where RUNX1 resides, resulting in a homozygous mutation with amplification via trisomy 21. (C-F) Variant allele frequency in four patients who progressed to leukemia. The size of the del(5q) clone was estimated with FISH analysis of MNC.

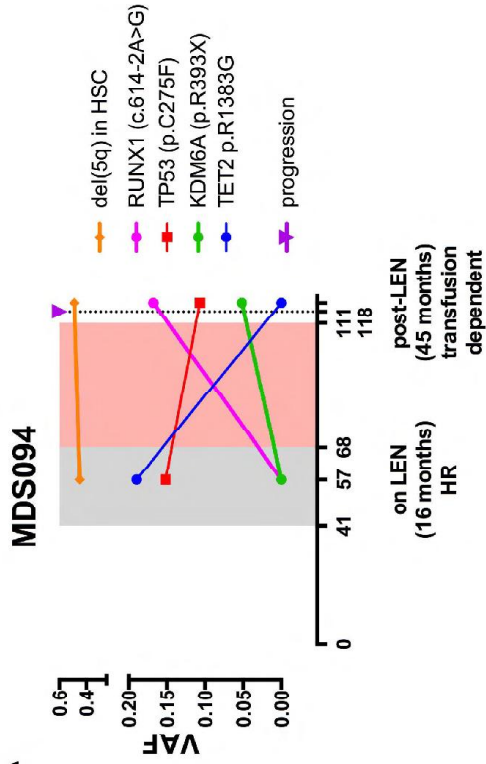
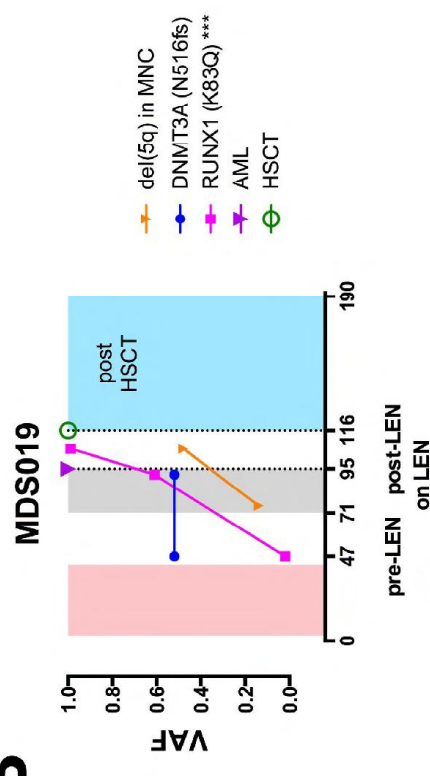
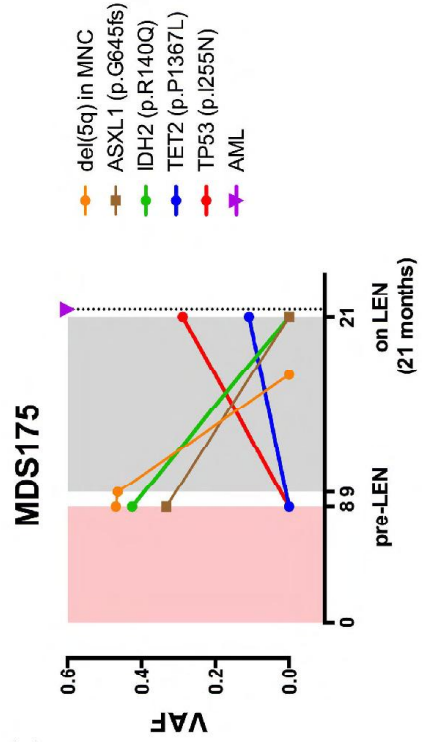
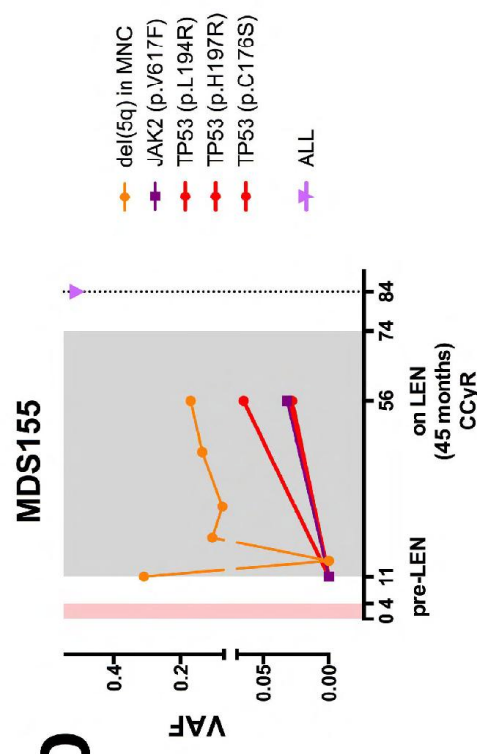
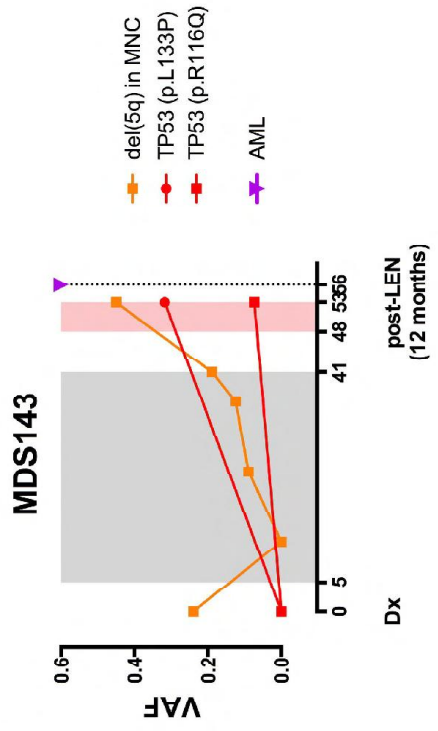
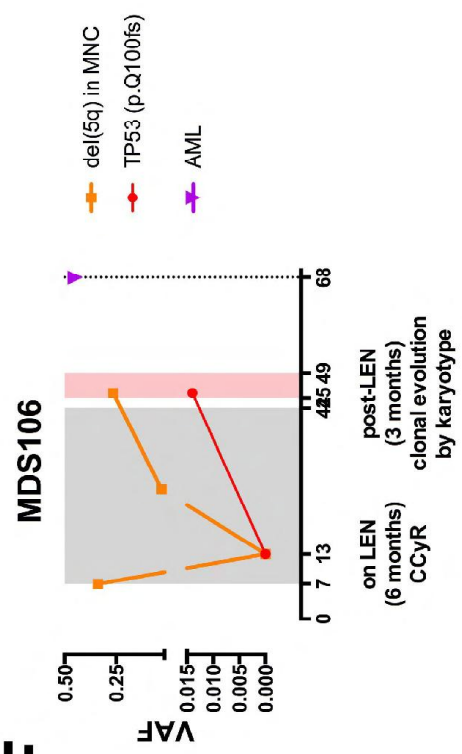
Figure 3. Detection of mutations in advance of clinical signs of progression. Depicted are the individual fates of 13 patients who progressed to either high-risk MDS (n=4) or leukemia (n=9). Shown are time of diagnosis, time point sequenced and whether mutation was detected or not (see legend).

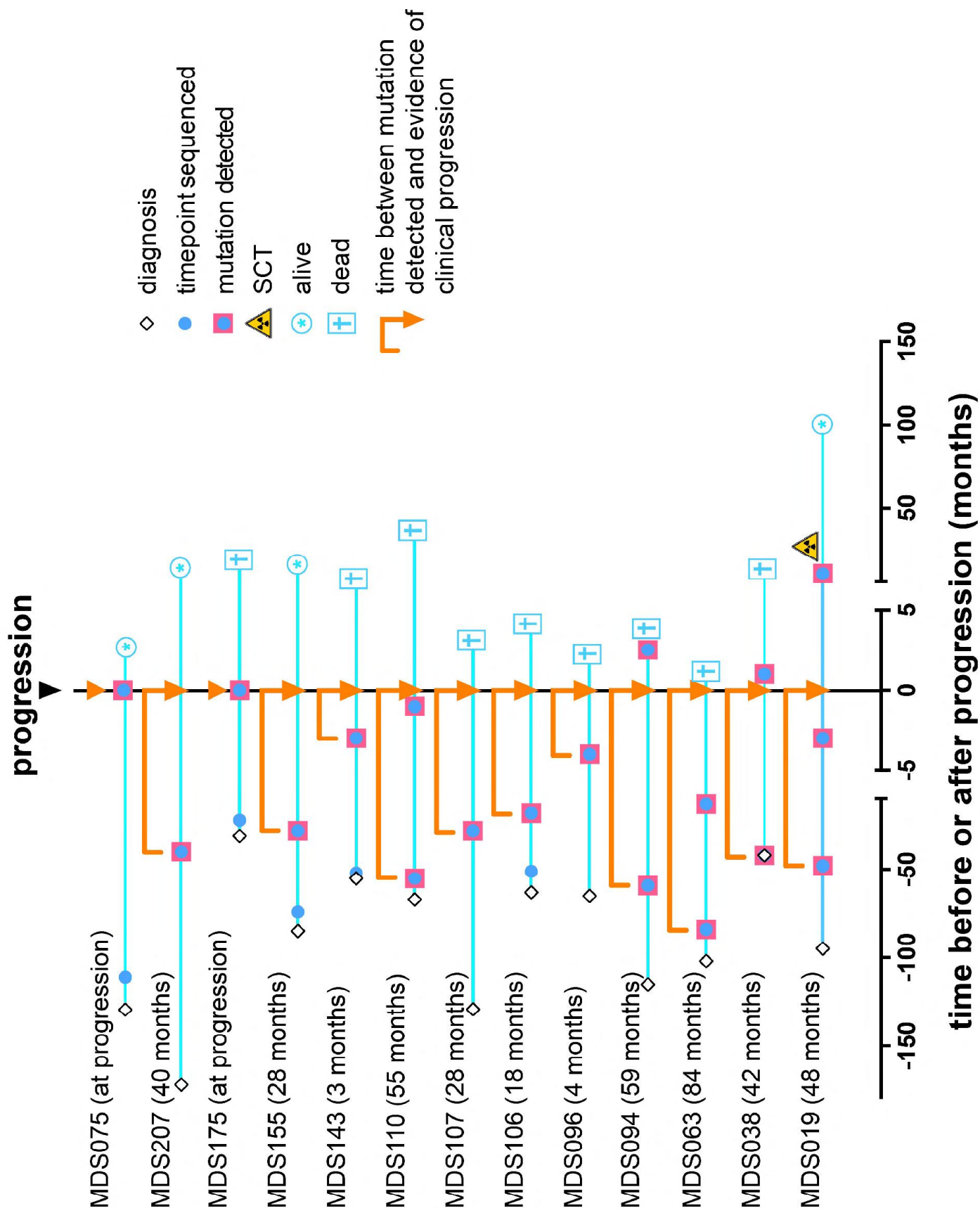
Figure 4. Surveillance of HSPC subsets and the phenotypic changes induced by lenalidomide. (A) FACS profiles of bone marrow stem and progenitor cells in normal age-matched control (top row), and representative del(5q) MDS at diagnosis (middle row), and del(5q) MDS treated with LEN. (B) Relative distribution of stem and progenitor cell subsets within lin-CD34+CD38- and lin-CD34+CD38+ compartments in normal controls and diagnostic/untreated del(5q), and LEN-treated del(5q). Indicated p-values are shown where significant by Mann-Whitney test. (C) Frequency within total BM and ratio of del(5q) versus normal HSC in serial samples of 4 patients (3 responders and one non-responder) during lenalidomide treatment and progression to AML. Abbreviations: TTP, time to progression; MNC, mononuclear cells; TD, transfusion-dependent; CR, complete response; LR, loss of response; PR, partial response).

Figure 5. Minor alterations within the microenvironment. (A) Heatmap of 13 genes associated with the HSC-niche interaction. The left six lanes show the healthy controls and the right six the del(5q) cases. (B) Immunohistochemistry for markers associated with niche cells in the bone marrow microenvironment. Shown are representative images from a normal control compared to one patient (MDS143) before LEN-treatment, during complete cytogenetic response (19 months on LEN, CCyR) and when the patient stopped responding to LEN (35 months).

whole cohort
n=35

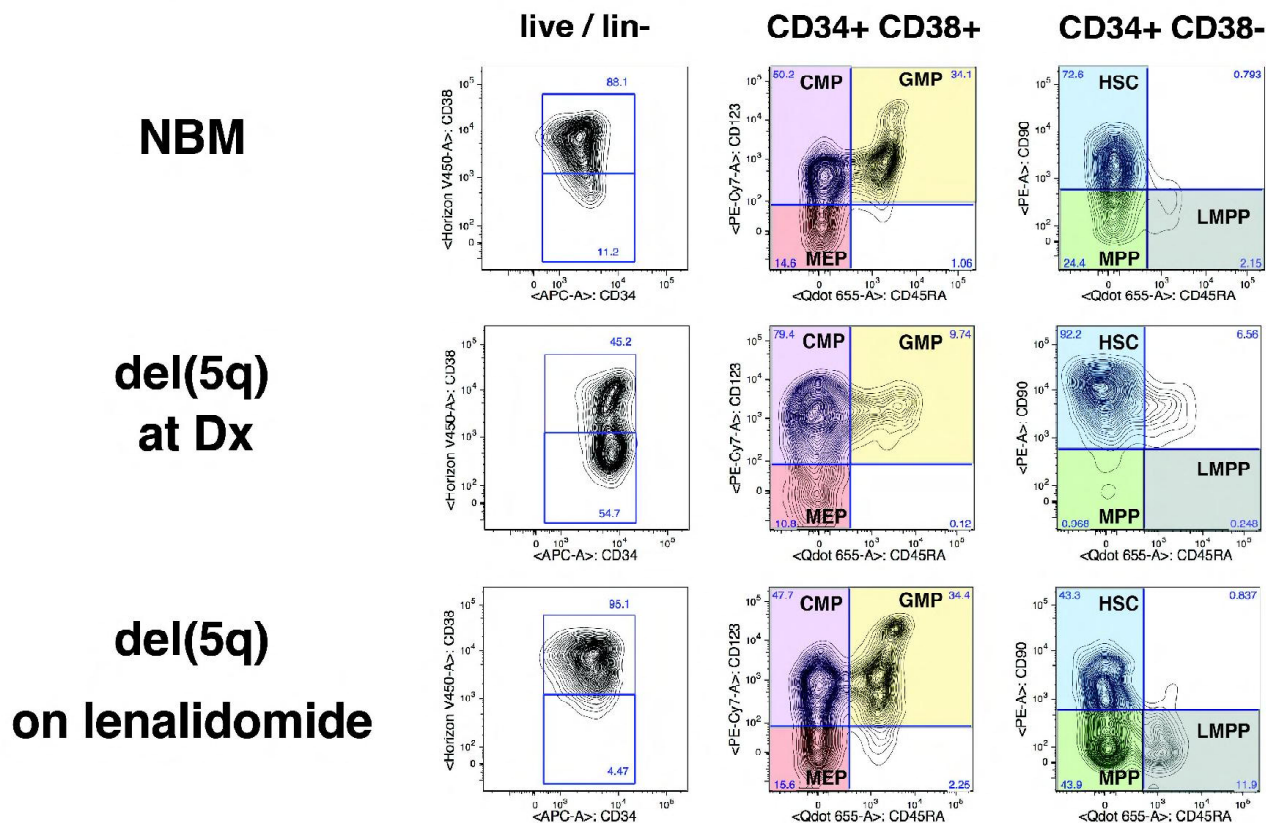


A**B****C****D****E****F**

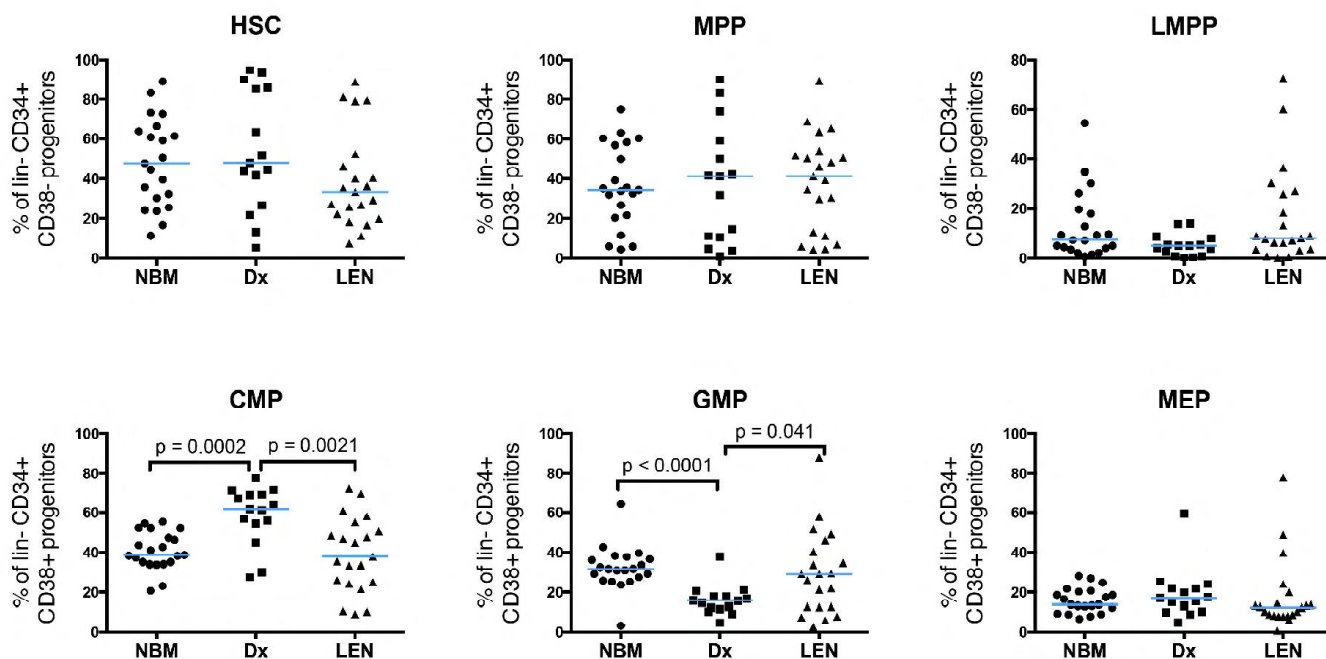


A

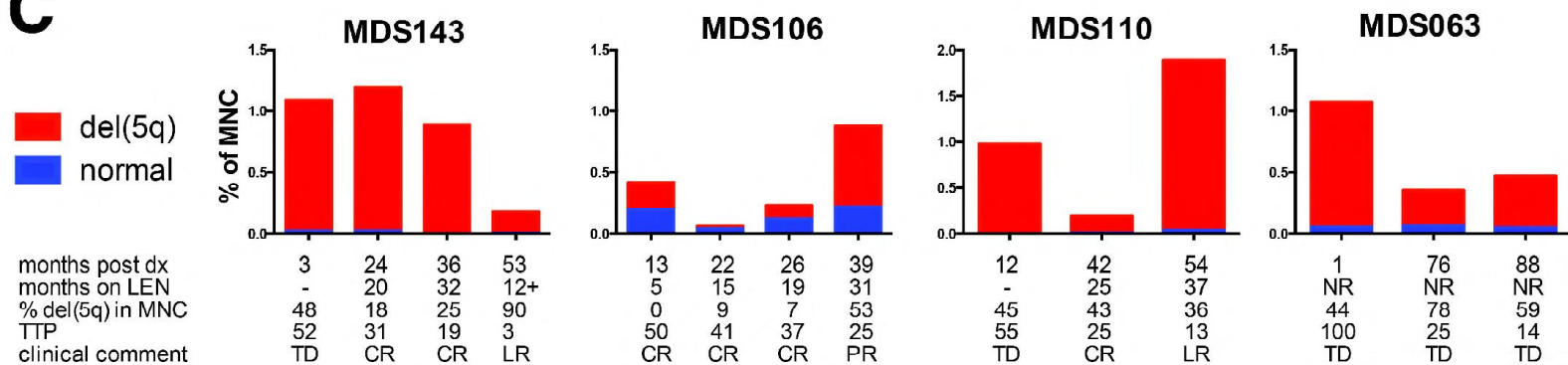
Sorting of stem cells and progenitors

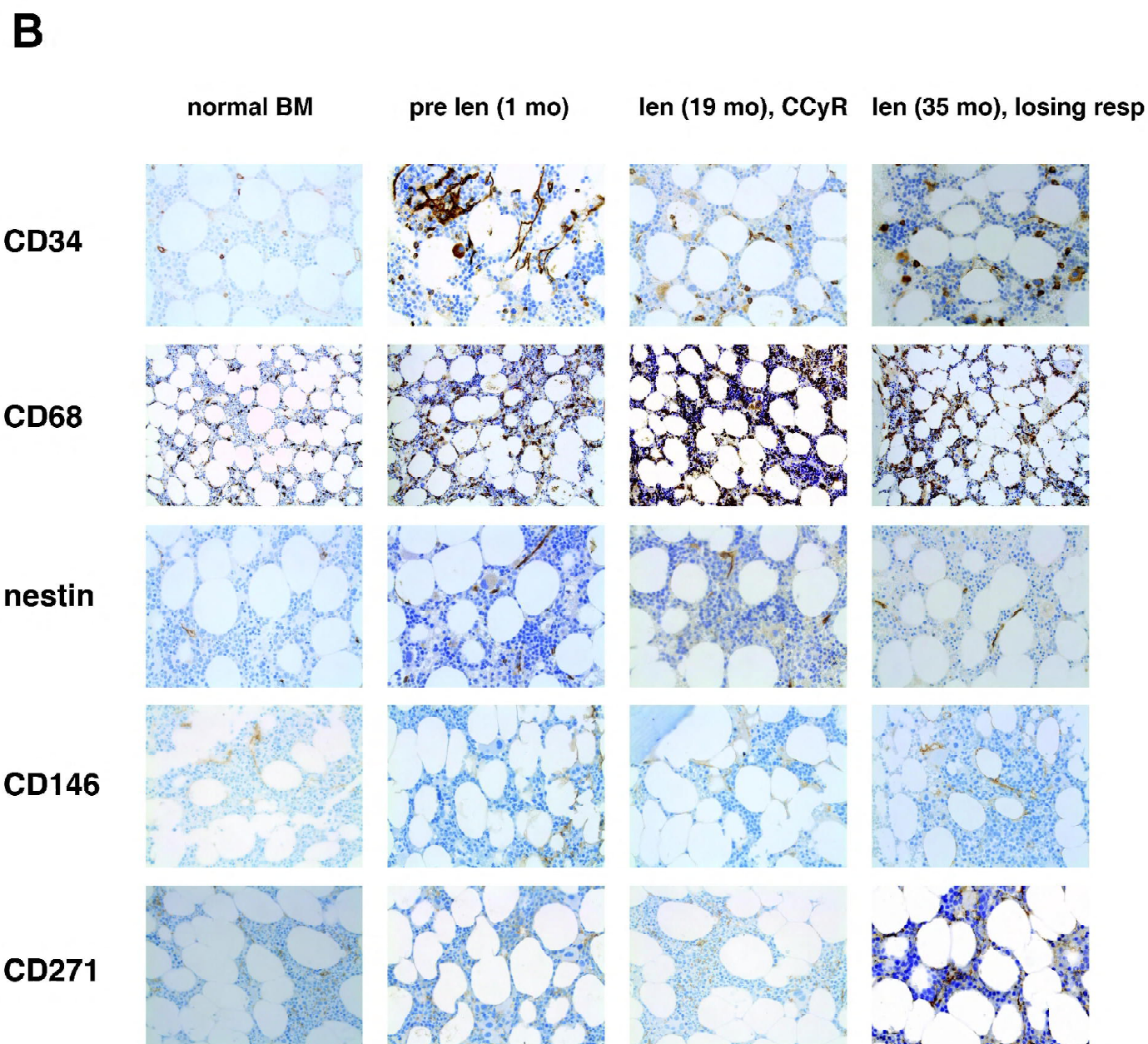
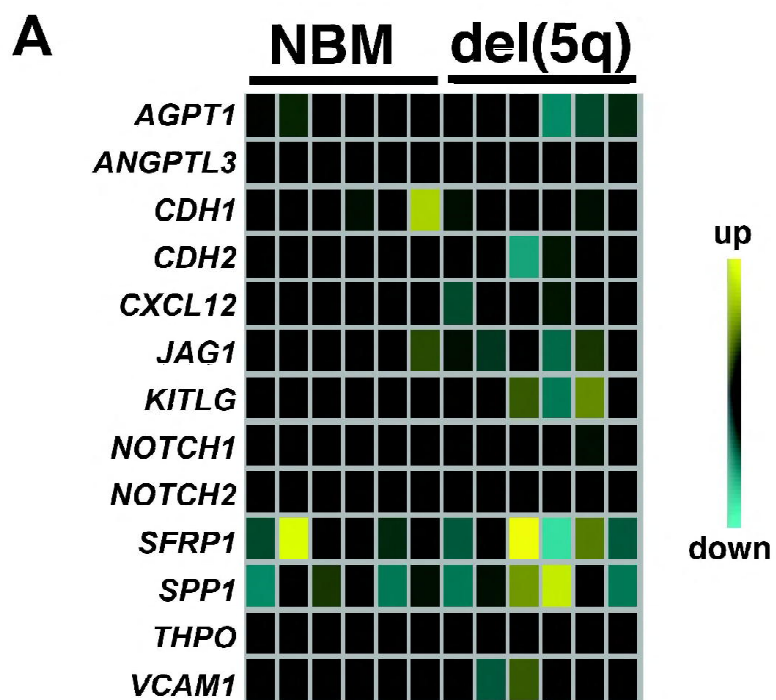


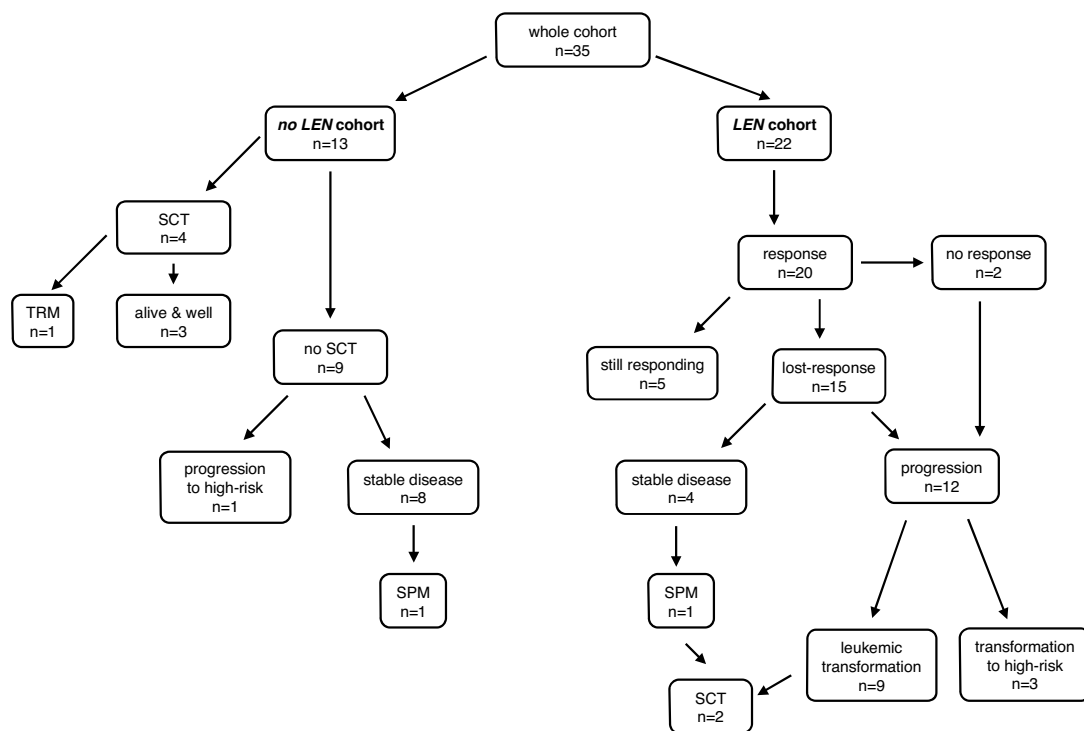
B



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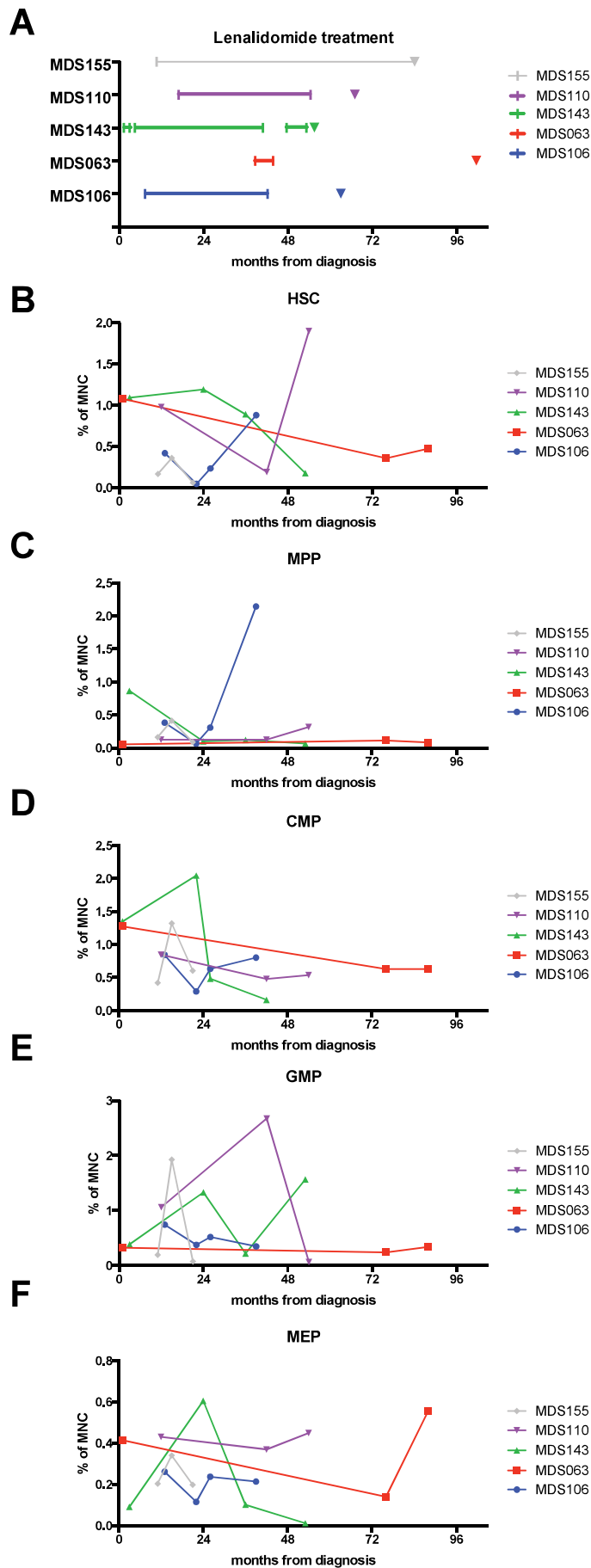




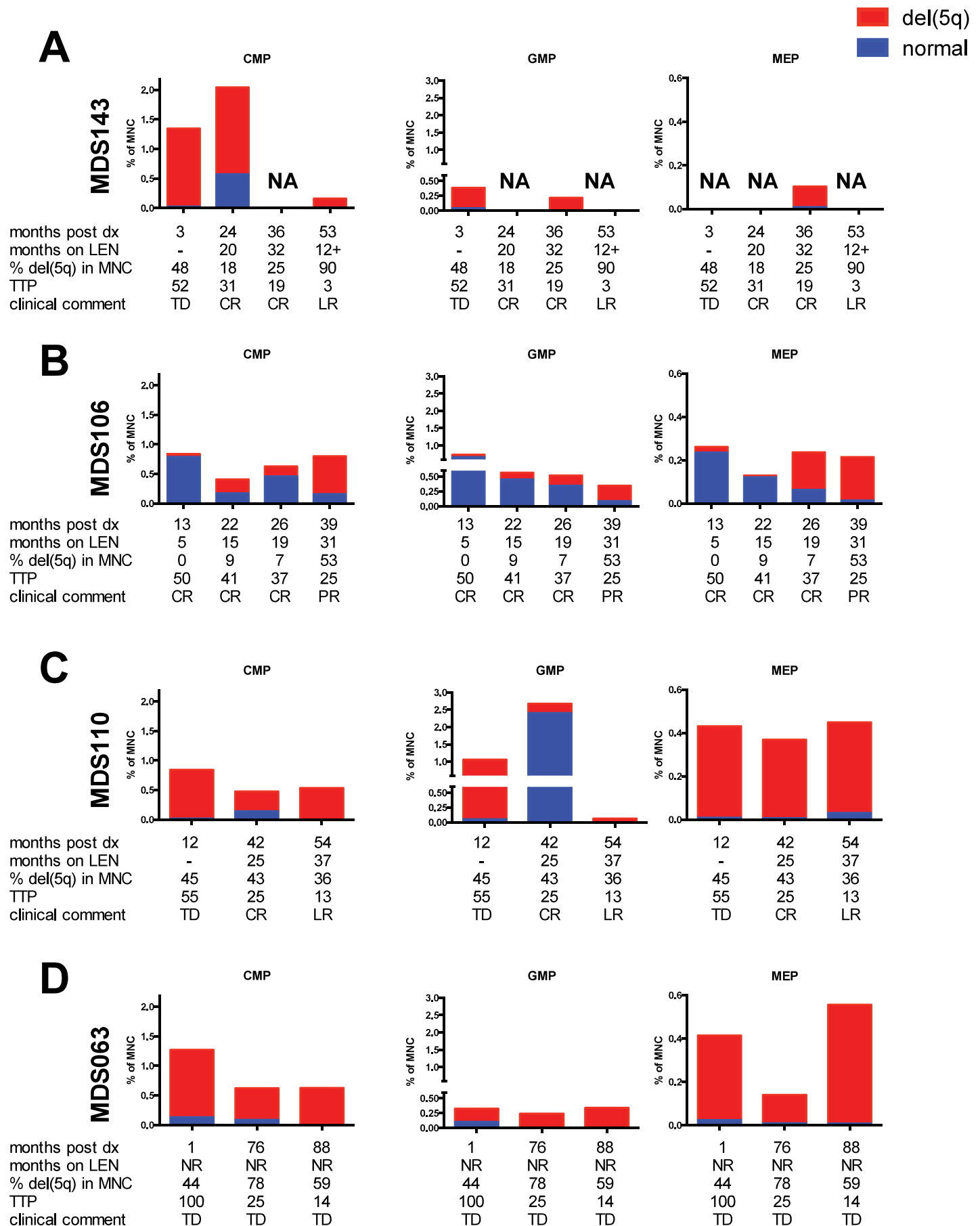
Supplementary Figure 1. A) Consort diagram of the cohort and description of the cohorts.
***SPM = secondary primary malignancy.**

Patient cohort not treated with LEN ('no LEN cohort'). Of thirteen patients (13/35, 37%) who did not receive LEN, only one patient (1/13, 8%) progressed to refractory anemia with excess blasts (RAEB-1) and no patient developed AML, eight patients (8/13, 62%) remained in stable phase, four of which received EPO-treatment. In this group, the median observation time from start of diagnosis was 31 months (range 0.5-187). The remaining four patients underwent allogeneic stem cell transplantation (SCT) during stable phase (INT-1 risk) at 45 to 72 years of age. Three of the nine non-transplanted patients have died (disease progression n=1, unrelated causes n=2). Of the four transplanted patients one died of transplantation-related mortality (TRM) while the other three patients are currently alive and well 30, 37 and 63 months post transplantation.

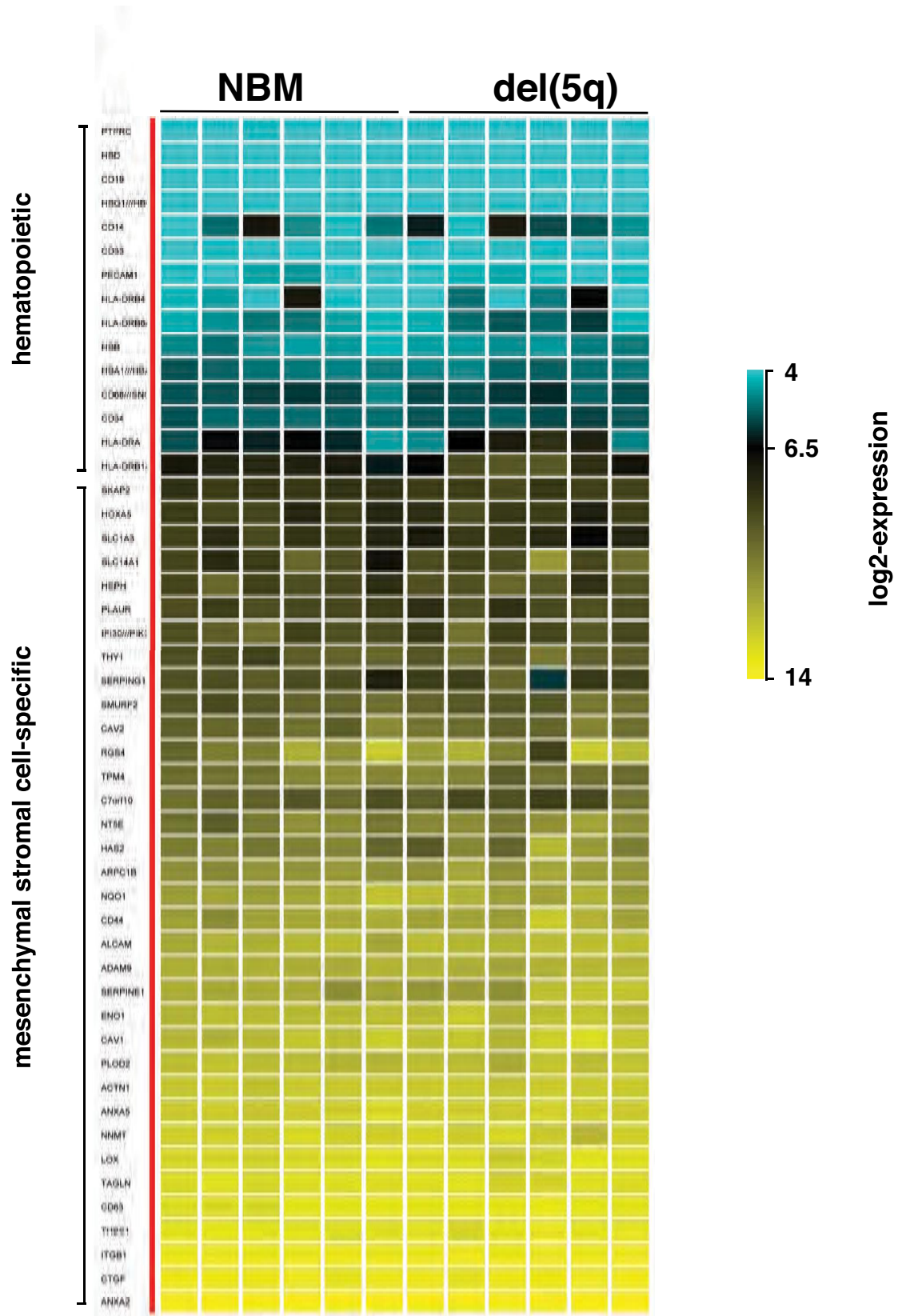
Patient cohort treated with LEN ('LEN cohort'). 22 patients (63%), who were transfusion dependent and either refractory to EPO or unlikely to respond based on high serum-EPO levels and transfusion intensity (Hellström-Lindberg, E., 1995. *British journal of haematology*, 89(1), pp.67–71) received LEN treatment. The median time from diagnosis to start of LEN was 20 months (range 0.5-82), the median duration of LEN treatment was 24 months (range 1-96), and the median observation time from start of LEN treatment was 55 months (range 19-129). Twenty of 22 patients (90%) showed at least a hematologic improvement to LEN according to the international guidelines (Cheson, B.D. et al., 2006. *Blood*, 108(2), pp.419–425), with a median response duration of 24 months (range 11-94). At the time of last follow-up, 5 patients (5/22, 23%) (MDS237, MDS239, MDS218, MDS489, and MDS538) were still responding 48, 37, 24, 20 and 19 months after start of LEN-treatment with a treatment duration of 1 to 24 months; two of these patients (MDS237 and MDS239) were treated for 11 and 1 months, respectively, and showed a continuous response despite being off LEN for more than two years. Amongst 17 patients who either lost their response to LEN or failed to respond upfront, twelve (12/17, 71%) progressed to higher-risk MDS (n=3) or leukemia (n=9; AML (n=8), ALL (n=1)) after a median of 76 months (range 31-184) post diagnosis, and 54 months (range 18-128) from start of LEN-treatment. Of the two patients who failed to respond to LEN, one patient (MDS063) discontinued LEN after 4 weeks due to poor compliance and no response, and developed AML 102 months after diagnosis, and 62 months after start of LEN. In the other non-responder (MDS224), LEN was discontinued after 3 months and the patient has currently stable disease receiving regular transfusions. Two patients underwent SCT, one was still responding to LEN but had developed breast cancer, the other patient was transplanted after transformation to AML and achieving complete remission after standard induction chemotherapy.



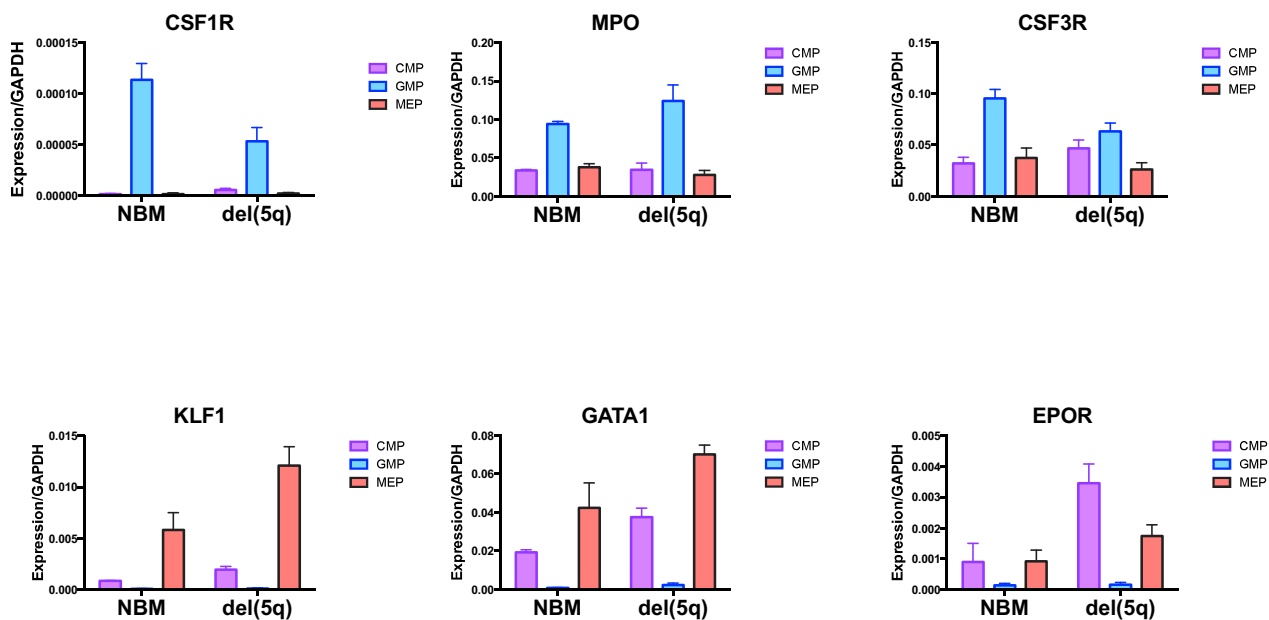
Supplementary figure 2 (related to Figure 4). Kinetic changes in HSPC subsets within the same patient over time during treatment and disease progression. A) shows the scheme for lenalidomide treatment. Triangle indicates time point of progression. B-F) Stem- and progenitor cell percentages in total BM for patients who initially responded to lenalidomide (n=4) or not (n=1). All patients shown eventually progressed to leukemia.



Supplementary figure 3 (related to Figure 4). Clonal size in the lin-CD34⁺ CD38 progenitor compartment.
 Abbreviations: TTP, time to progression; MNC, mono-nuclear cells; TD, transfusion-dependent;
 CR, complete response; LR, loss of response; PR, partial response; NR, no response



Supplementary Figure 4 (related to Figure 5). Heatmap of raw expression values of MSC-related genes in MSC samples. Expression of several hematopoietic genes are shown as a control. The left six lanes show the 5q- cases and the right six the healthy controls. The color bar indicates log2 expression values.



Supplementary Figure 5. Mean (SEM) expression of myeloid and erythroid transcripts within CMPs, GMPs and MEPs from normal BM (n=6) and del(5q) MDS (n=8).

Supplementary Table 1. Genes studied using 2 haloplex gene panels.
note that the 74 gene panel includes all the genes in the 42 gene panel.

haloplex-42	haloplex-74	
ASXL1	APC	NOTCH1
BCOR	ASXL1	NPM1
CBL	ATRX	NRAS
CEBPA	BAP1	NXF1
CSF3R	BCOR	PDGFRB
DNMT3A	BCORL1	PDS5B
EPOR	BRAF	PHF6
ETV6	CBL	PRPF40B
EZH2	CEBPA	PTEN
FLT3	CREBBP	PTPN11
GATA1	CSF3R	RAD21
GATA2	CTCF	RB1
GATA3	CTNNA1	RIT1
IDH1	CUX1	RPS14
IDH2	DIS3	RUNX1
JAK2	DNMT3A	SETBP1
KDM6A	ELANE	SF1
KIT	EP300	SF3A1
KRAS	EPOR	SF3B1
MLL	ETV6	SH2B3
MPL	EZH2	SMC1A
NPM1	FAM5C	SMC3
NRAS	FLT3	SRSF2
PDS5B	GATA1	STAG1
PRPF40B	GATA2	STAG2
RAD21	GATA3	STAT5B
RUNX1	GNAS	TET2
SF1	HNRNPK	TP53
SF3A1	IDH1	U2AF1
SF3B1	IDH2	U2AF2
SH2B3	IKZF1	WT1
SMC1A	IRF1	ZRSR2
SMC3	JAK2	
SRSF2	JAK3	
STAG1	KDM6A	
STAG2	KIT	
TET2	KRAS	
TP53	LUC7L2	
U2AF1	MLL	
U2AF2	MPL	
WT1	MYC	
ZRSR2	NF1	

Supplementary Table2. Individual patient samples analyzed and mutations detected.

Sample ID	at Dx or before any treatment	Time after diagnosis (months)	Treatment	months on LEN	clin status at sampling	Clinical outcome	Gene	Mutation	Variant allele ratio
MDS038	yes	0	none		TD		TP53	p.Y163C	0.20
MDS038	no	19	LEN	3	CHR		TP53	p.Y163C	0.21
MDS038	no	28	LEN	11	CHR		TP53	p.Y163C	0.14
MDS038	no	47	none		RAEB-2	AML	TP53	p.Y163C	0.23
MDS063	yes	18	none		TD		MLL	p.E3013Q	0.48
							PTPN11	p.E69V	0.33
MDS063	no	88	none		progression	AML	MLL	p.E3013Q	0.50
							PTPN11	p.E69V	0.39
							TP53	p.C176Y	0.01
MDS094	no	57	LEN	16	CHR		TET2	p.R1383G	0.19
							TP53	p.C275F	0.15
MDS094	no	118	none		progression	RAEB-2 & ovarial Ca	KDM6A	p.R393X	0.05
							TP53	p.C275F	0.11
MDS106	no	13	LEN	5	CCyR		no mutation		
MDS106	no	45	none		progression	AML	TP53	p.Q100fs	0.01
MDS124	yes	1	none		TD		No Mutation		
MDS124	no	44	HSCT		post SCT	SCT	No Mutation		
MDS143	yes	3	none		TD		No Mutation		
MDS143	no	52	none		progression	AML	TP53	p.L265P	0.32
							TP53	p.R116Q	0.07
MDS155	yes	11	None		TD		No Mutation		
MDS155	no	72	LEN	61	CCyR	ALL	JAK2	p.V617F	0.03
							TP53	p.L194R	0.06
							TP53	p.H179R	0.03
							TP53	p.C176S	0.03
MDS175	yes	8	none		TD		ASXL1	p.G645fs	0.33
							IDH2	p.R140Q	0.43
MDS175	no	31	LEN	21	progression	AML	TET2	p.P1367L	0.11
							TP53	p.I255N	0.29
MDS185	yes	114	none		TD		DNMT3A	p.S669fs	0.09
MDS185	no	155	none		post SCT	SCT	No Mutation		
MDS218	yes	9	none		TD		ASXL1	p.G645fs	0.06
MDS218	no	50	EPO		TD	still responding	ASXL1	p.G645fs	0.03
							CSF3R	p.T781fs	0.27
							CUX1	p.N579fs	0.31
MDS224	yes	20	none		TD		DNMT3A	p.F354fs	0.37
MDS224	no	48	none		TD	SD	DNMT3A	p.F165fs	0.33
MDS237	yes	48	none		TD		No Mutation		
MDS237	no	95	none		CCyR	still responding	No Mutation		
MDS335	yes	0	none		TD		No Mutation		
MDS335	no	19	EPO		CHR	SD	No Mutation		
MDS019	yes	47	none		TD		DNMT3A	N516fs	0.52
							RUNX1	K83Q	0.02
MDS019	no	92	LEN	24	CHR		DNMT3A	N516fs	0.52
							RUNX1	K83Q	0.61
MDS019	no	106	none		AML	AML & SCT	DNMT3A	N516fs	0.50
							RUNX1	K83Q	0.99
MDS110	yes	12	none		TD		SF3B1	p.K700E	0.18
							TET2	p.K1439fs	0.10
MDS110	no	66	none		progression	AML	NRAS	p.G12V	0.43
							SF3B1	p.K700E	0.44
MDS075	no	70	LEN	14	CHR	SD	DNMT3A	p.R729Q	0.08
							DNMT3A	p.R899Afs	0.24
MDS075	no	184	none		progression	AML	MYD88	p.L273P	0.06
							TET2	p.H1325fs	0.23
							TP53	p.R273C	0.29
							BCOR	p.A1496P	0.05
MDS032	no	86	LEN	46	CHR	breast ca & SCT	EZH2	p.V568fs	0.95
							ASXL1	p.A946fs	0.45
MDS076	no	42	LEN	26	PHR	SD	No Mutation		
MDS096	no	61	LEN	14	PHR	RAEB-1	DNMT3A	p.R882H	0.35
							RUNX1	p.S303X	0.27
							SRSF2	p.P95L	0.61
							TET2	p.R1262Q	0.37
MDS107	no	100	LEN	67	CHR	RAEB-1	RUNX1	p.P200fs	0.40
							TET2	p.S1107X	0.48
							TET2	p.R1261H	0.44
							ASXL1	p.R634fs	0.32
							ZRSR2	p.E246X	0.91
MDS117	no	9	LEN	7	CCyR	SD	ASXL1	p.Q780X	0.09
MDS153	yes	1	none		SD	SD & SPM	No Mutation		
MDS207	yes	132	EPO		SD	RAEB-1	EZH2	p.R34X	0.06
							TP53	p.R141H	0.80
MDS233	yes	0	none		SD	SD	ASXL1	p.R634fs	0.06
							SETBP1	p.D868N	0.15
MDS239	yes	0	none		SD	still responding	KDM6A	p.H1357L	0.47
MDS240	yes	0	none		SD	SD	No Mutation		
MDS355	yes	0	none		SD	SD	No Mutation		
MDS359	yes	0	none		SD	SCT	JAK2	p.V617F	0.06
							TP53	p.R267W	0.22
MDS432	yes	0	none		SD	SD	SF3B1	p.K700E	0.11
MDS445	yes	0	none		TD	SD	TET2	p.L431X	0.35
MDS489	yes	0	none		SD	still responding	DNMT3A	p.R736C	0.29
MDS499	yes	0	none		TD	SCT	DNMT3A	p.731_732del	0.40
MDS526	yes	0	none		TD	SD	DNMT3A	p.I655T	0.12
							SF3B1	p.K700E	0.30
MDS538	yes	0	none		TD	still responding	No mutation		
MDS555	yes	0	none		SD	SD	BCOR	p.Q231fs	0.03
							TP53	p.P278A	0.22
							TP53	p.V104A	0.18

TD = transfusion-dependent

CHR = complete hematologic response

CCyR = complete cytogenetic response

PHR = partial hematologic response

SD = stable disease

Supplementary Table3. Clinical details of samples used for surveillance of HSPC subsets by flow cytometry.

Patient ID	months post diagnosis	sex	WHO	Karyotype	IPSS	Hb	WBC	ANC	BM blasts	transfusion	%del(5q) of MNC by FISH	treatment lenalidomide	treatment response
MDS063	1	Female	5q-	46,XX,del(5)(q13q33) [9], 44-45,XX,del(5)(q13q33) [3], 46,XX [13], 44-45,XX [2]	Low	95	9.1	6.1	4	no	44	pre	NA
MDS063	76	Female	5q	46,XX,del(5)(q13q33)[11]/45,XX,-5[3]/45,XX,del(5)(q13q33),-5[1]/46,XX[11]	Low	116	7.6	4.3	4	yes	78	NA	NR
MDS063	88	Female	5q	46,XX,del(5)(q13q33)[16]/46,XX,idem,del(17)(p?13)[9]	Low	93	8.1	4.1	5	yes	59	NA	NR
MDS106	13	Male	5q-	NA	Low	127	4.9	3	4.5	yes	0	on (5 mo)	CCyR
MDS106	22	Male	5q-	47,XY,+21[4]/47,XY,del(5)(q13q33),+21[2]/46,XY[21]	Int-1	126	11	8.6	5.5	no	9	on (15 mo)	CHR
MDS106	26	Male	5q-	NA	Int-1	130	3.4	1.7	0	no	7	on (19 mo)	CHR
MDS106	39	Male	5q	47,XY,+21[3]/47,XY,del(5)(q13q33),+21[12]/46,XY[9]	Low	103	4.1	2.2	3	no	53	on (31 mo)	PR
MDS110	12	Female	5q	46,XX,del(5q)/9[25]	Int-1	89	3.5	1.6	2	no	45	pre	TD
MDS110	42	Female	5q	46,XX,del(5)(q13q33)[20]/46,XX[5]	Low	110	4.9	2.3	2	no	43	on (25 mo)	CHR
MDS110	54	Female	5q	46,Xxdel(5)(q13q33)[20]/46,XX[5]	Low	107	6.9	3.8	3.5	yes	36	on (37 mo)	LR
MDS143	3	Male	5q	46 XY, del(5)(q13q33) [14], 46 XY [13]	Low	89	3.3	2	5	yes	48	pre	TD
MDS143	24	Male	5q	NA	Low	148	23	8	3	no	18	on (20 mo)	CHR
MDS143	36	Male	5q	46,XY,del(5)(q13q31)[6]/46,XY[19]	Low	127	3.2	1.8	3	no	25	on (32 mo)	CHR
MDS143	53	Male	5q	46,XY,del(5)(q12q33)[11]745-47,XY,del(5)(q12q33),del((7)(q22)), -11,-17,-18,-20,-21,+3-7mar[cp8]/46,XY[8]	Int-1	90	3.5	1.8	9.5	yes	90	post (12 mo)	LR
MDS155	11	Female	5q-	46,XX,del(5)(q13q33) [24]/46,XX[4]	Low	114	3.4	2.3	1.5	yes	62	pre	TD
MDS155	15	Female	5q-	NA	Low	146	4.7	2.9	4.5	no	0	on (4 mo)	CCyR
MDS155	21	Female	5q-	NA	Low	129	3.3	1.3	3.5	no	21	on (11 mo)	CHR

Abbreviations: TD = transfusion-dependent
 CHR = complete hematologic response
 PR = partial response
 LR = loss of response
 CCyR = complete cytogenetic response

Supplementary Table 4. Comparison of TP53 analysis by IHC against targeted sequencing and deep sequencing

PAT ID	months post dx	% TP53 by deep seq	TP53+ by IHC	type of TP53 by targeted sequencing	% TP53 by targeted seq
MDS143	3	NA	0	no mut	0
MDS143	12	0	0	no material available	
MDS143	24	NA	0		
MDS143	41	NA	2		
MDS143	53	NA	8	L265P and R116Q	32 and 7
MDS143	55	NA	25		
MDS094	20	NA	1	no material available	
MDS094	60	NA	3	C275F	15
MDS094	72	29	5.5	no material available	
MDS094	91	NA	5		
MDS094	103	NA	15		
MDS094	115	NA	11		
MDS094	118	NA	10	C275F	11
MDS175	0	NA	4	NA	NA
MDS175	9	NA	4	other mut	0
MDS175	10	NA	11	NA	NA
MDS175	14	0	4	NA	NA
MDS175	32	NA	50	I255N	29
MDS106	13	NA	0	no mut	0
MDS106	26	NA	0	no material available	
MDS106	39	0	NA		
MDS106	45	NA	2	Q100fs	1
MDS063	0	NA	0	no material available	
MDS063	18	NA	NA	other mut	0
MDS063	22	NA	0	no material available	
MDS063	34	0	0		
MDS063	76	NA	5		
MDS063	88	NA	7	C176Y	1
MDS075	34	NA	0	no material available	
MDS075	70	0	0		
MDS075	184	NA	10	R273C	29
MDS038	0	20	1	no material available	
MDS038	3	NA	2		
MDS038	4	NA	7		
MDS038	19	21	10		
MDS038	28	14	1		
MDS038	47	23	10		
MDS038	55	NA	10.5		
MDS155	21	NA	10	no material available	
MDS155	29	NA	10		
MDS155	57	NA	12	L194R, H179R and C176S	6, 3 and 3
MDS359	0	NA	6	R267W	22
MDS207	111	NA	5	NA	
MDS207	132	NA	<5	R131W	80
MDS207	172	NA	20	NA	
MDS555	10	NA	NA	P278A and V104A	22 and 18