Progression in patients with low- and intermediate-1-risk del(5q) myelodysplastic syndromes is predicted by a limited subset of mutations

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ABSTRACT

A high proportion of patients with lower-risk del(5q) myelodysplastic syndromes will respond to treatment with lenalidomide. The 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 starting treatment. The 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 cell 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 seven of nine 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 hematopoietic stem and progenitor cells 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 cases of del(5q) myelodysplastic syndromes, whether the detection of such mutations before and after lenalidomide treatment 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 that del(5q) HSC are 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 patients with del(5q) MDS, in whom 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 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 2 years[9] and approximately 40% of the patients in the MDS 004 study had progressed to acute myeloid leukemia 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 the patients

Between 2004 and 2015 we included consecutive patients at the Karolinska University Hospital in Stockholm, Sweden based on the following criteria: MDS with a low- or intermediate-1-risk score at diagnosis according to the International Prognostic Scoring System 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 purify HSPC, by fluorescence activated cell sorting (FACS), 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 that used by Jadersten *et al.*[12] Besides defining "progression" as the development of acute myeloid leukemia (9 cases), four cases were defined as having progression based on the acquisition of additional karyotypic abnormalities (n=3) and an increase of marrow blasts from <5% to 11% (n=1) in combination with worsening cytopenias.

Flow cytometry, cell sorting and analysis of gene expression

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⁺); and MEP (lin⁻CD34⁺CD38⁺CD123⁻CD45RA⁻). Cell populations were isolated from CD34-enriched normal and MDS mononuclear cells by FACS on a FACS Aria and used for subsequent analyses. Gene expression was analyzed by Fluidigm Dynamic Arrays as previously described[3] (*Online Supplementary Figure S5*).

Fluorescence *in situ* hybridization

Flow-sorted cell populations were spun onto glass slides. Slides were subsequently treated with pepsin and fixed with formaldehyde/MgCl$_2$. The LSI EGR1/D5S721, D5S23 Dual Color Probe (Abbott-Vysis, Downers Grove, IL, USA) was used to detect deletions of 5q31; 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 fluorescence *in situ* hybridization (FISH) analysis does not detect additional cytogenetic changes, standard cytogenetic studies were performed on mononuclear cells taken at the same time-points.

Bone marrow morphology and immunohistochemistry

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). Bone marrow cellularity and fibrosis were assessed according to European consensus guidelines.[15] Immunohistochemistry was performed for different markers including p53 DO-1 (Santa Cruz, Biotechnology, Inc., USA),[11] CD34, CD68, Nestin, CD271, CD146 (Novocastra, UK), using the automated Bond™ and Ventana Bench Mark XT systems according to the manufacturers' instructions. Microvascular density was quantified as the number of blood vessels per high power field, using regular light microscopy at high (400x) magnification as previously described.[16] Blood vessels were identified as CD34⁺ 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 described.[17]

Mesenchymal stromal cell cultures and RNA isolation

Mesenchymal stromal cells (MSC) were isolated from six untreated del(5q) cases and six healthy volunteers using a previously published standard procedure[18] and expanded as detailed elsewhere, while uniformly fulfilling the minimal MSC criteria.[19] 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 mesenchymal stromal cells

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 labeled with the 3' IVT Express Kit (Affymetrix, Santa Clara, CA, USA) following the manufactur-

er's recommendations. Biotin-labeled 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 16 h in a 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 algorithm.[22] Data were analyzed using GeneSpring 12.6 (Agilent Technologies). Quality control results obtained for scale factors, background levels, percentages of present calls, 3'/5' *GAPDH* ratio, and intensities of spike hybridization controls were within the acceptable ranges for all samples.[23]

DNA sequencing and bioinformatics analyses

Haloplex target enrichment for Illumina (Agilent) was applied for mutation screening in panels of either 42 or 74 frequently mutated genes (*Online Supplementary Table S1*) according to the manufacturer's instructions. Of note, the 42-gene panel covers most genes reported to be recurrently mutated in MDS[4-6] 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 a GeneElute DNA extraction kit (Sigma) and quantified by Qubit. All samples were individually barcoded using 96 barcoding oligos by Agilent during enrichment and the quality of individual libraries was checked by Tape Station D1K assays (Agilent). Sequencing was performed on pooled samples using either a HiSeq 2000 (Illumina) sequencer through paired–end, 100 bp reads or a 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 variants were identified using VarScan 2 in mpileup2cns mode. Variants were annotated using ANNOVAR.[24] An ECD DNA control included in the Haloplex kit was used to filter out sequencing errors. Variants were selected for further analysis if they met the following criteria: (i) minimum coverage of 100X, (ii) minimum of 20 variant reads, (iii) having a variant allele frequency of >0.03 for all genes except for *TP53* for which the limit of detection was set at ≥0.01 based on our previous studies that demonstrated these detection levels to be clinically relevant,[11,12,25] (iv) not present in the 1000 Genome database, (v) not listed in dbSNP unless listed in the COSMIC 65 database, (vi) truncating or damaging based on SIFT if not present in COSMIC 65. Sequencing results for case MDS019 were obtained by exome capture performed using SureSelect Human All Exon 50 Mb (Agilent), with sequencing done on an Illumina Genome Analyzer IIx platform. Sequencing results for case MDS110 have already been published.[2]

Statistical analysis

Survival and time to progression (defined as blast increase to >10% or acquisition of a complex karyotype) were updated in April 2016 and were measured from the time of diagnosis. Continuous variables were compared using the Mann-Whitney U-test or Student t test, as appropriate. Categorical variables were analyzed by the Fisher exact test. All statistical calculations were performed using Graph Pad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Patients' outcome

We analyzed 35 patients with del(5q) MDS, with low- or intermediate-1-risk disease according to the International Prognostic Scoring System, at one or more time-points by targeted sequencing (*Online Supplementary Tables S1* and *S2*). Patients were allocated to receive lenalidomide treatment (n=22) or not (n=13) based on the severity of their anemia, co-morbidities and availability of other therapeutic options, such as allogeneic stem cell transplantation. After a median observation period of 61 months (range, 0.5-187) from diagnosis, 17 patients remained alive. The median survival of all patients (median age 82 years, range, 45-96) was 102 months (range, 0.5-187). These patients included six who underwent allogeneic stem cell transplantation at 145, 135, 116, 42, 12 and 6 months after diagnosis, with five of these six transplanted 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 *Online Supplementary Figure S1*.

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 *Online Supplementary Table S2*. We found no differences in other clinical parameters (e.g., age, blood counts, or additional cytogenetic abnormalities) between patients in whom we detected recurrent mutations and those in whom we did not. Considering all time-points for 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 seen in high-risk MDS patients.[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 two groups (*P*>0.99). Only two out of 22 patients failed to respond to lenalidomide treatment and although both of these patients harbored mutations, meaningful statistical analysis of these two patients was not possible.

Of the 35 patients, 13 (37%) progressed to high-risk MDS (refractory anemia with excess blasts-1, n=3 and refractory anemia with excess blasts-2, n=1) or leukemia (n=9) at a median of 85 months (range, 31-184) after diagnosis. Of the 27 patients for whom diagnostic or pre-treatment samples were available, nine (33%) showed no mutations, while 18 (67%) had one or more mutation (*Online Supplementary Table S1*). The presence of any recurrent mutation covered by these MDS panels early in the patients' 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, an absence of mutations was suggestive of freedom from progression (*P*=0.073).

For 16 patients, material was available from more than one time-point, enabling longitudinal assessment of allelic burden in relation to treatment with lenalidomide or stem

cell transplantation. 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 earli-

est sample in only one case (MDS038) and acquired in six cases (MDS063, 075, 106, 143, 155, 175). Overall, there was a strong correlation between the detection of a TP53 mutation by targeted sequencing and cells staining strongly positive for *TP53* by immunohistochemistry (*Online Supplementary Table S4*). Importantly, of six patients with evidence of acquisition of mutations in *TP53* by targeted sequencing, five had been analyzed by deep sequencing without evidence of mutation at the diagnostic time-point.[12] Of these, four were negative for TP53 by immuno-histochemistry. Interestingly, however, the fifth patient

Figure 1. The mutational spectrum in del(5q) patients differs in untreated *versus* lenalidomide-treated patients. (A) Study outline and clinical fate of patients untreated ('no LEN' cohort) or treated with lenalidomide ('LEN' cohort). *denotes two patients who are alive and well after stem cell transplantation (SCT). (B) Spectrum of mutations in relation to clinical outcome in LEN-treated *versus* untreated patients.

who was negative by deep sequencing did actually show positive immunohistochemical 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 at which samples were taken from these patients. Patient MDS110 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 refractory anemia with excess blasts-2 in the cohort not treated with lenalidomide 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 developed subsequently, testing positive for any of

them carried a high probability (13/16, 81%) of 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 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 hematopoietic stem and progenitor cell subsets under lenalidomide therapy

In order to investigate the impact of lenalidomide ther-
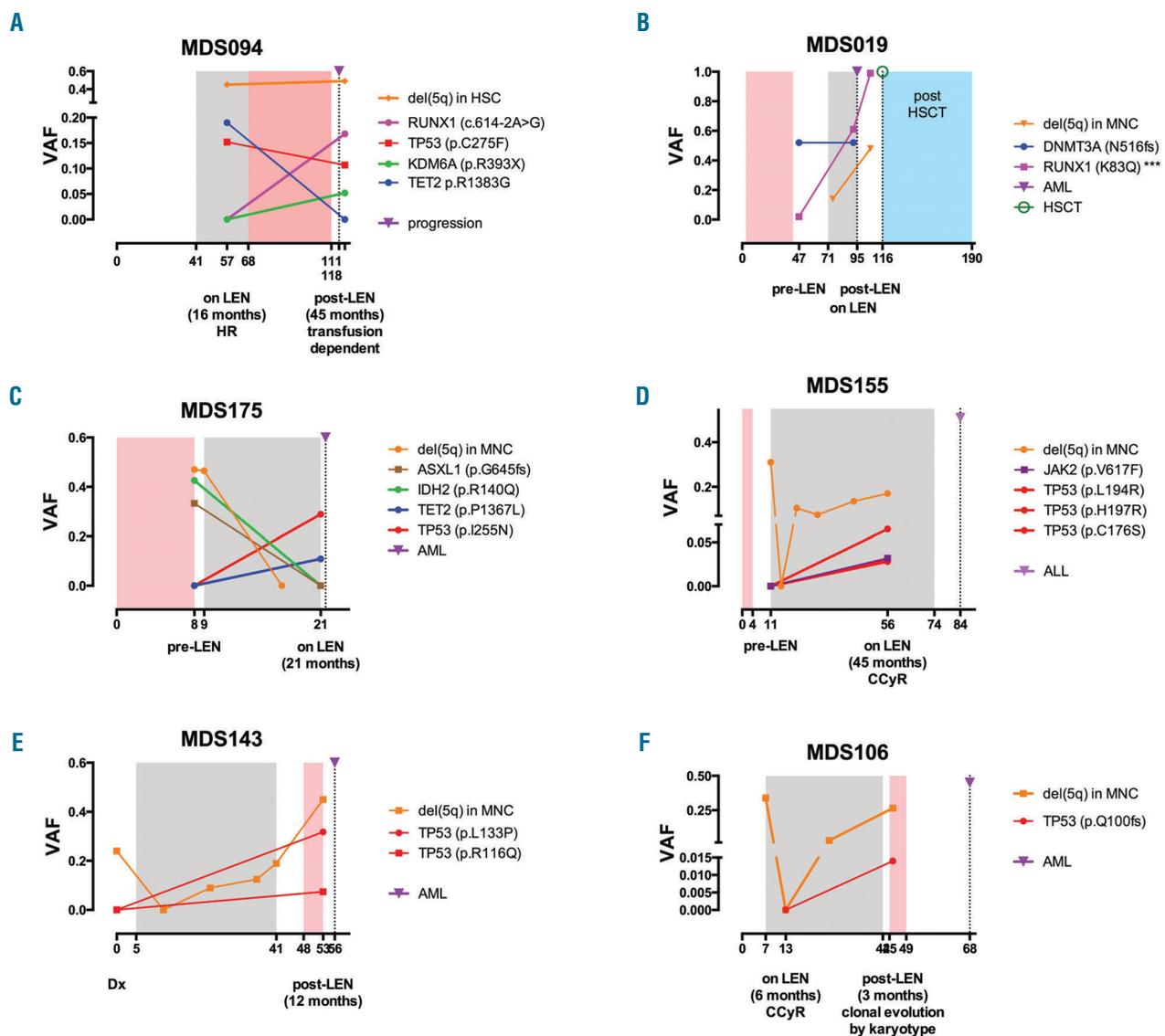
Figure 2. Longitudinal assessment of mutations during treatment with erythropoietin (shaded in red) and lenalidomide (shaded in gray). (A) Frequency of mutations in relation to the del(5q) clone in a patient who progressed to high-risk disease. (B) Variant allele frequency in a patient who progressed to leukemia, received induction therapy and went into complete remission and was transplanted. *** This patient had trisomy 21, the region in which *RUNX1* resides, resulting in a homozygous mutation with amplification via trisomy 21. (C-F) Variant allele frequencies in four patients who progressed to leukemia. The size of the del(5q) clone was estimated with fluorescence *in situ* hybridization analysis of mononuclear cells. VAF: variant allele frequency; LEN: lenalidomide; MNC: mononuclear cells; HSC: hematopoietic stem cells; HSCT: hematopoietic stem cell transplantation; AML: acute myeloid leukemia; ALL: acute lymphocytic leukemia; CCyR: complete cytogenetic response.

apy on distinct HSPC in del(5q) MDS patients, we performed multicolor flow-cytometry. The distribution of sub-populations within the Lin⁻CD34⁺CD38⁻ compartments, including HSC, MPP and LMPP,[2,13] remained similar in del(5q) MDS patients, both at diagnosis and during lenalidomide treatment, compared to that in 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 CMP. Upon lenalidomide treatment, the GMP and CMP distribution reverted to frequencies comparable to those of normal Lin⁻CD34⁺CD38⁺ cells.

Serial samples were available for five patients who progressed to leukemia, including one patient who did not respond to lenalidomide (MDS063). This allowed us to monitor kinetic changes in HSPC subsets within the same patient over time during treatment and disease progression (Figure 4C, *Online Supplementary Figure S2* and *Online Supplementary Table S3*). 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 *Online Supplementary Figure S3*). 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, the myeloid progenitor subsets (CMP, GMP and MEP) also showed minimal clonal involvement (*Online Supplementary Figure S3B*), 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) HSC 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 HSPC or if extrinsic, microenvironmental 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 typical gene signatures for MSC (*Online Supplementary Figure S4*). However, we found no genes expressed in a statistically significant different manner ($P<0.05$, Welch t-test and Benjamini–Hochberg multiple testing correction), even when specifically looking for genes previously implicated in HSC-niche interactions[29] (Figure 5A).

We next investigated bone marrow biopsies in healthy
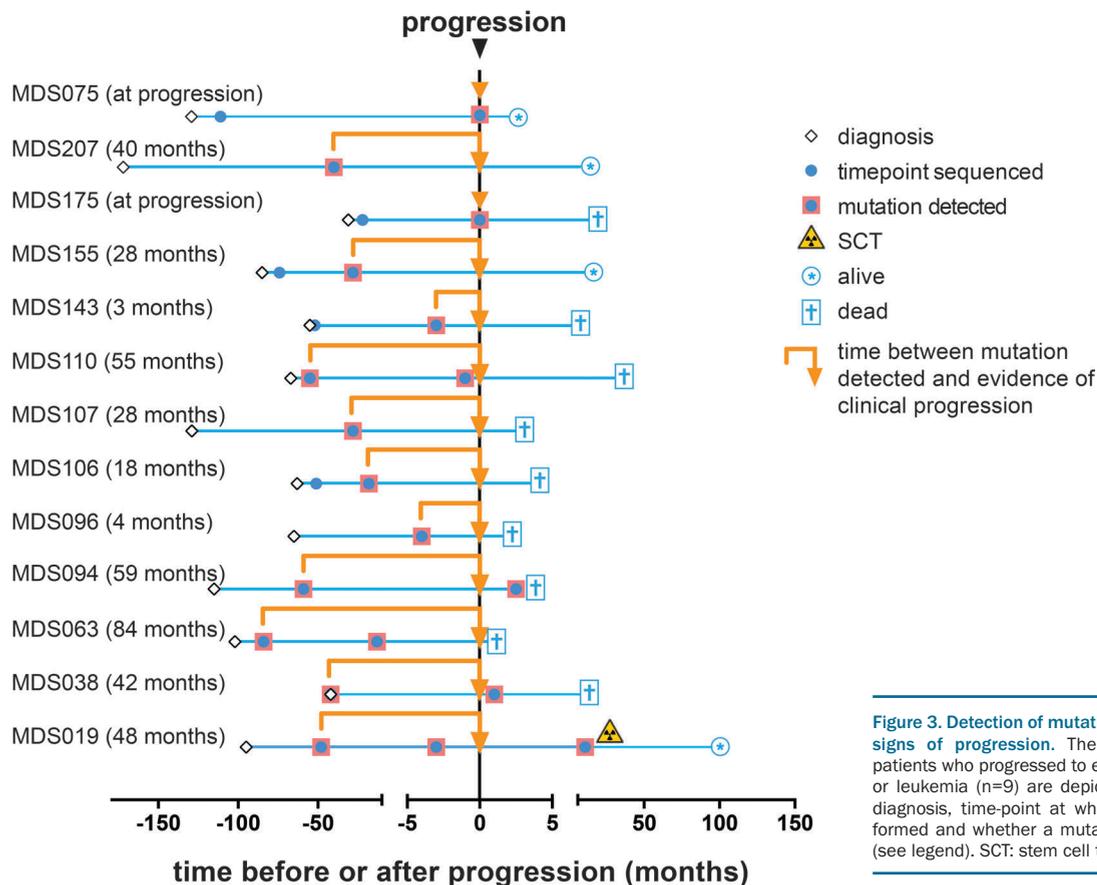
Figure 3. Detection of mutations in advance of clinical signs of progression. The individual fates of 13 patients who progressed to either high-risk MDS (n=4) or leukemia (n=9) are depicted showing the time of diagnosis, time-point at which sequencing was performed and whether a mutation was detected or not (see legend). SCT: stem cell transplantation.
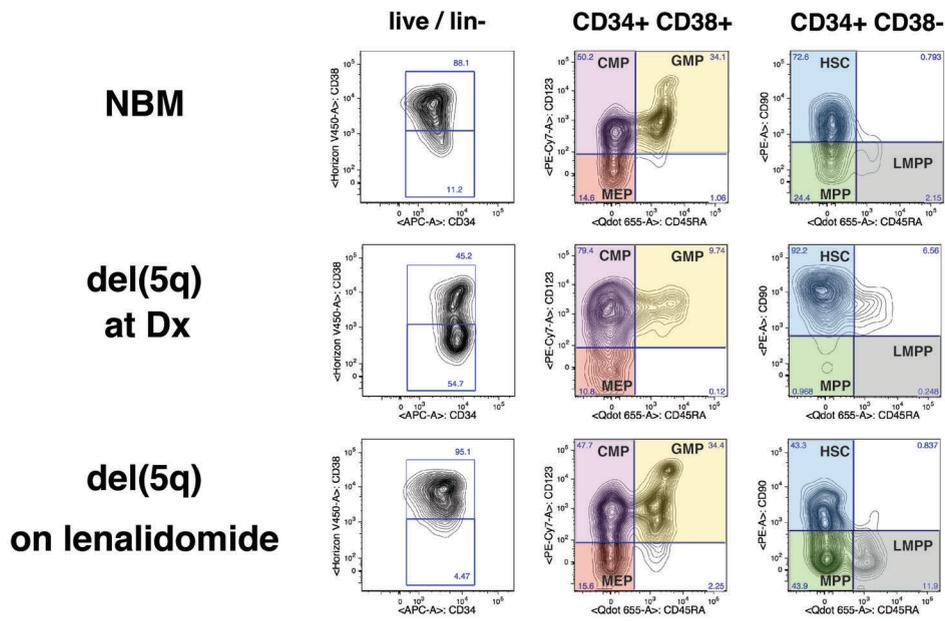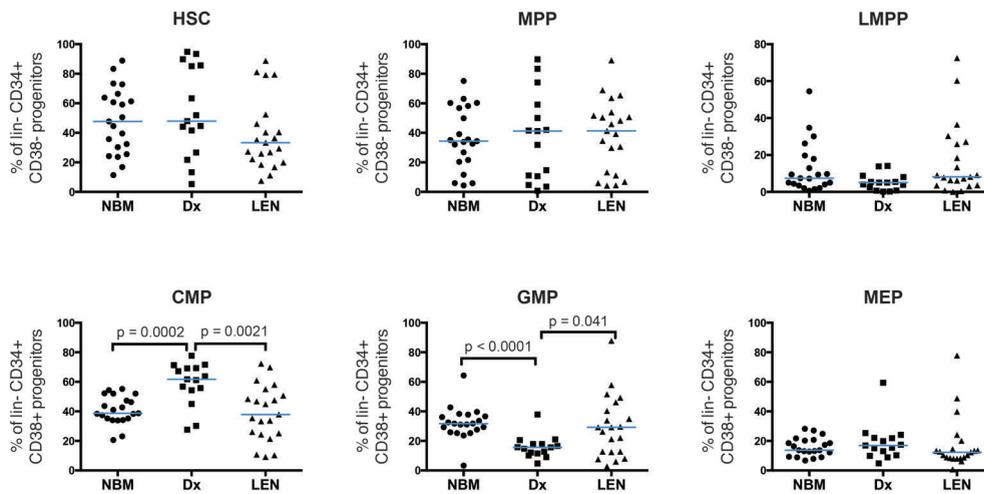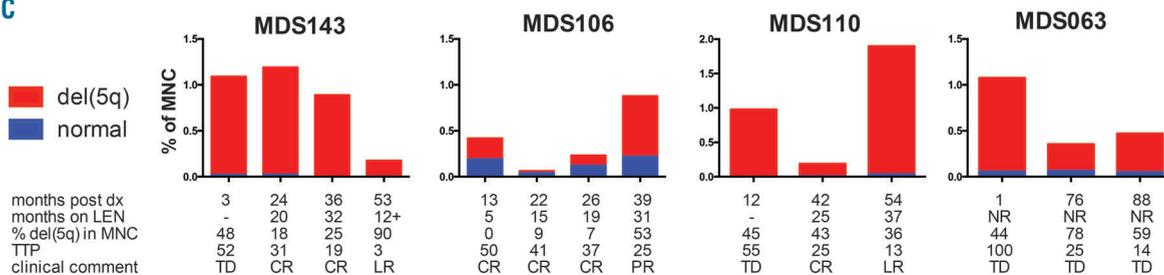
Figure 4. Surveillance of hematopoietic stem and progenitor cell subsets and the phenotypic changes induced by lenalidomide. (A) FACS profiles of bone marrow stem and progenitor cells in a normal age-matched control (top row), and a representative case of del(5q) myelodysplastic syndrome at diagnosis (middle row), and del(5q) myelodysplastic syndrome treated with lenalidomide. (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 lenalidomide-treated del(5q). Indicated *P*-values are shown when significant by the Mann-Whitney test. (C) Frequency within total bone marrow and ratio of del(5q) *versus* normal HSC in serial samples of four patients (3 responders and 1 non-responder) during lenalidomide treatment and progression to acute myeloid leukemia. NBM: normal bone marrow; Dx: diagnosis; LEN: lenalidomide; TTP: time to progression (months); MNC: mononuclear cells; TD: transfusion-dependent; CR: complete response; LR: loss of response; PR: partial response.

controls and in del(5q) MDS patients before and during lenalidomide treatment. Material for longitudinal analysis by immunohistochemistry was available for five patients. These analyses revealed that microvessel density was significantly higher in del(5q) MDS than in normal controls (microvessel density values of 5.2±3.2 *versus* 2.4±1.2, respectively; *P*=0.02) but decreased during the initial phase of lenalidomide treatment in all five patients analyzed (Figure 5B). Subsequent therapeutic failure was associated with an increase in bone marrow cellularity and microves-

sel density in four of the five patients. The number of CD68+ macrophages was not increased in bone marrow samples from del(5q) MDS patients as compared to the number in controls; however, upon lenalidomide treatment a decrease was noted which was paralleled by a decrease in cellularity (Figure 5B). Surrogate markers for MSC (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
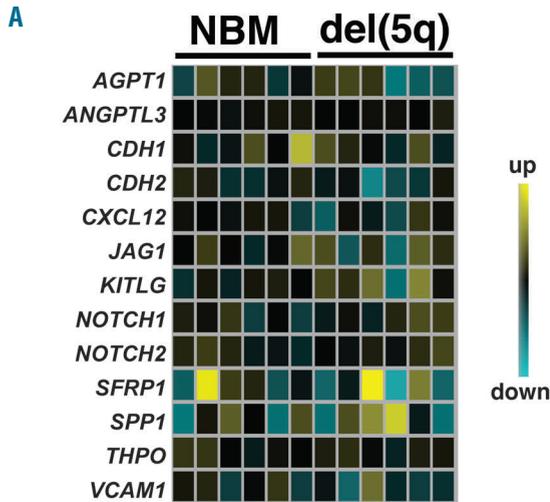
Figure 5. Minor alterations within the microenvironment. (A) Heatmap of 13 genes associated with the hematopoietic stem cell-niche interaction. The left six lanes show the healthy controls (NBM) and the right six the del(5q) cases. (B) Immunohistochemistry for markers associated with niche cells in the bone marrow microenvironment. Representative images from a normal control (normal BM) compared to one patient (MDS143) before lenalidomide-treatment, during complete cytogenetic response (19 months on lenalidomide, CCyR) and when the patient stopped responding to lenalidomide (35 months). LEN: lenalidomide; CCyR: complete cytogenetic response; resp: response.
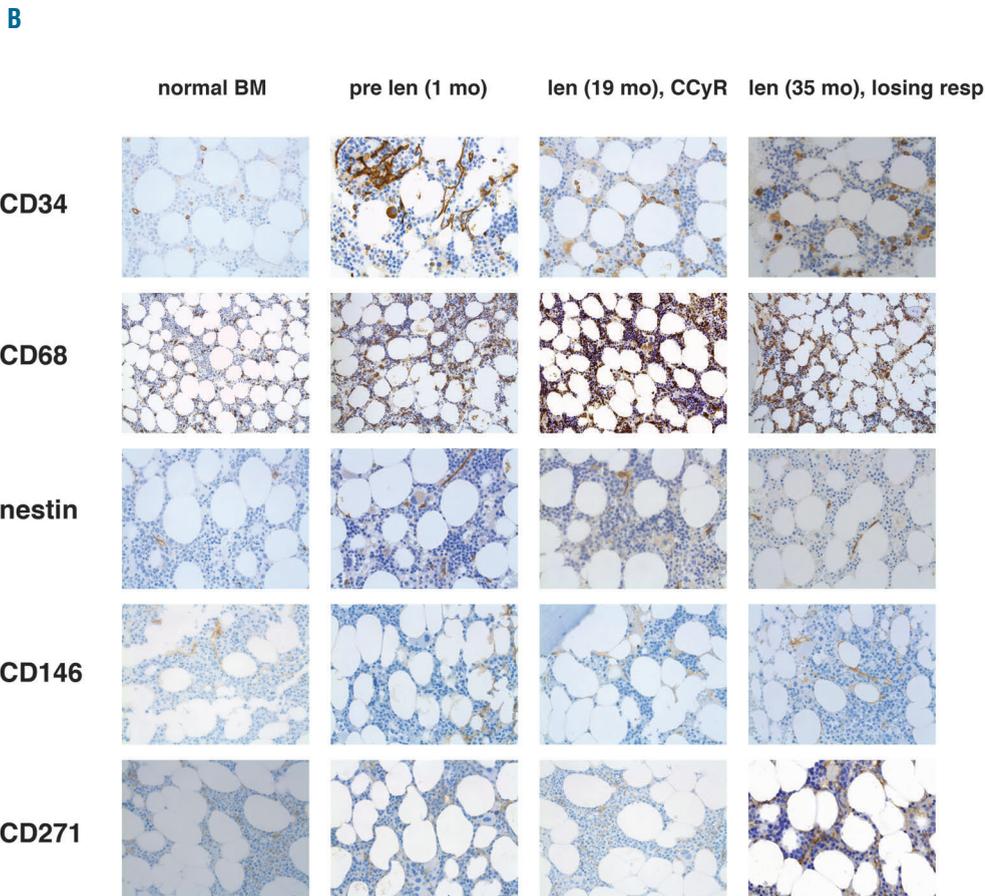
despite affecting microvessel density, lenalidomide did not exhibit its effects primarily via alteration of the cellular composition of the microenvironment based on the MSC markers tested.

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 5q deletion. 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 at the latest time-point analyzed, and several patients showed increased allele burdens and gains 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 next-generation sequencing data from 94 non-del(5q) lower-risk patients treated with lenalidomide ± erythropoietin and found that only about one-third of these patients had more than one genetic event, most often consisting of a *SF3B1* mutation plus one additional mutation. Moreover, response to lenalidomide was associated with a decrease in allelic burdens of the identified mutations, and only two of 18 patients analyzed at a later time-point had acquired new recurrent mutations.[30]

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 before 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 prior to treatment cannot be excluded, but five of these six patients had previously been analyzed in a study by Jädersten *et al.* using deep-sequencing analysis (coverage of 1200X) and had been found to be negative,[12] and four also proved to be TP53-negative by immunohistochemistry 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 5q deletion. 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* acute myeloid leukemia.[32-34]

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 three 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 acute myeloid leukemia.[35-37] Furthermore, we found mutations in *TET2* in six of 13 patients with evidence of disease progression. Although three 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] although one study reported that *TET2* mutations were associated with shorter survival in MDS patients undergoing HSC transplantation.[42] Our data on del(5q) patients are in line with recent findings in myeloproliferative neoplasms in which *TET2* mutations were associated with disease progression if they were acquired in a *JAK2*-mutated subclone.[43]

Our study of the clonal dynamics of all major HSPC *in vivo* shows that clonal advantage is not 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, among 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 cells.[44] 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 MDS.[45] 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 MSC. While seemingly at odds with recent findings in cohorts of multiple subtypes of MDS,[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 conducting a prospective study aimed at investigating, in a larger number of del(5q) MDS cases before and after 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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