Clonal heterogeneity in the 5q- syndrome: p53 expressing progenitors prevail during lenalidomide treatment and expand at disease progression

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ABSTRACT

Clonal heterogeneity has not been described in patients with myelodysplastic syndrome with isolated del(5q), for which lenalidomide has emerged as a highly potent treatment. However, transformation to acute myeloid leukemia is occasionally observed, particularly in patients without a cytogenetic response to lenalidomide. We performed molecular studies in a patient with classical 5q- syndrome with complete erythroid and partial cytogenetic response to lenalidomide, who evolved to high-risk myelodysplastic syndrome with complex karyotype. Immunohistochemistry of pre-treatment marrow biopsies revealed a small fraction of progenitors with overexpression of p53 and sequencing confirmed a *TP53* mutation. *TP53* mutated subclones have not previously been described in myelodysplastic syndrome with isolated del(5q) and indicates a previously unknown heterogeneity of this disease. The aberrant subclone remained stable during the treatment with lenalidomide and expanded at transformation, suggesting that this pre-existing cell population had molecular features which made it insensitive to lenalidomide and prone to disease progression.

Key words: resistance, del(17p), thalidomide analog.

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Introduction

The immunomodulatory drug lenalidomide is a potent treatment for low-risk myelodysplastic syndrome (MDS) with a karyotype including the deletion of 5q31 (del[5q]), with 67% achieving transfusion independency, and 45% obtaining complete cytogenetic remission.[1] However, 50% of responders relapse within two years, and the estimated risk of leukemic transformation at ten years is 15% for cytogenetic responders and 67% for patients without a cytogenetic response.[2]

Design and Methods

Patients and treatment

A patient with classical 5q- syndrome (MDS with isolated del[5q] according to the WHO 2008 classification)[3] received lenalidomide within a clinical trial (Celgene MDS-004). The clinical and laboratory studies were approved by the Ethical Committee for Research at the Karolinska Institutet, Stockholm, Sweden. Informed consent was obtained from the patient.

Bone marrow assessment and immunohistochemistry

Histopathology and morphology were assessed on consecutive bone marrow (BM) samples. Immunohistochemistry on paraffin-embedded sections was performed using mouse monoclonal antibodies: p53 (clone DO-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA); CD34 (DakoCytomation, Glostrup, Denmark). Ten normal BMs were stained and demonstrated p53 expression in less than 0.01% of cells.

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Chromosome banding analysis

After 20-48 h of culture, metaphases of BM were prepared and fluorescence R-banding was performed.[4] Karyotypes were described according to the International System for Human Cytogenetic Nomenclature.[5]

Fluorescence in situ hybridization (FISH) and M-FISH

Interphase FISH was performed using a probe for the locus 5q31.[6] Depending on the cytogenetic aberrations detected, probes (Abbott, Wiesbaden, Germany) were applied for the *MLL*-locus (11q23), *RB1*-locus (13q14), *TP53*-locus (17p13), *BCL2*-locus (18q21), and *AML1*-locus (21q22). At least 200 nuclei were evaluated. M-FISH analysis was carried out as previously described.[6]

Cells and cultures

BM sampling for *in vitro* culture was performed pre-lenalidomide and at time of disease progression. $CD34^+$ progenitors were selected from BM mononuclear cells and cultured for seven days ± lenalidomide 10 μM, as recently described.[7]

Gene expression profiling and gene sequencing

RNA and DNA were extracted from cultured progenitors at day 7. Gene expression profiling analysis was performed as described elsewhere.[7,8] Pathway analysis was performed using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA, USA). DNA-sequences spanning exons 5-8 of *TP53* were amplified by polymerase chain reaction (PCR), using published primer sequences.[9] PCR products were sequenced using the BigDye Terminator v1.1 kit (Applied Biosystems).

Results and Discussion

Clinical observations

A 76-year old woman was diagnosed with 5q- syndrome in April 2004. She was transfusion-dependent up-front (2 units of red blood cells per month), and had a transient response to EPO. Lenalidomide treatment was started in February 2006, leading to a complete erythroid response, and a reduction in the del(5q) clone from 75% to 25% by FISH. G-CSF was given during the first months. The transfusion-need recurred following pneumonia in October 2007; however, neutrophil and platelet counts were still normal. In November 2007, 22 months after start of treatment, BM examination showed 9% blasts and cytogenetic analysis demonstrated a large clone with complex karyotype, including del(5q). By FISH, a loss of the *TP53* locus at 17p13 and

Table 1. Summary of clinical data and laboratory investigations.

Δt*	Clinical	WHO‡ status†	Karyotype	FISH 5q§	FISH additional	PLT ($x10^9$)	ANC ($x10^9$)	Blasts	CD34+	NPMc+	p53+
-21	Primary diagnosis	5q-	46,XX,del(5)(q14q34)[24] 46,XX[2]	ND	13q14(RB1x2) 96% 17p13(P53x2) 97%	482	1.8	2%	<1%	5%	2%
-5	RBC transfusions	5q-	46,XX,del(5)(q14q34)[24] 46,XX[1]	85%	11q23(MLLx2) 100% 13q14(RB1x2) 100% 17p13(P53x2) 99%	450	2.3	4%	2%	5%	4%
0	Pre-LEN sampling‡	5q-	46,XX,del(5)(q14q34)[21] 46,XX[5]	76%	ND	369	1.9	<5%	ND	ND	ND
4	LEN, CER, on G-CSF	5q-	ND	ND	ND	135	1.3	2%	2%	5%	1%
8	LEN, CER, on G-CSF	5q-	46,XX,del(5)(q14q34)[9] 46,XX[16]	25%	ND	207	3.1	ND	ND	ND	ND
14	LEN, CER, on G-CSF	5q-	46,XX,del(5)(q14q34)[14] 46,XX[12]	32%	ND	197	3.2	ND	ND	ND	ND
22	LEN, loss of response, transformation	RAEB-1	46,XX,del(5)(q14q34)[10] 46,idem,+10,der(11)t(11;16)(p11;?),der(11;17)(p10;q10),der(16), t(11;16)(q12;q12), -18[3] 47,idem,+10,der(11)t(11;16)(p11;?),der(11;17)(p10;q10),+13,der(16)t(11;16)(q12;q12),-18[6] 48,idem,+10,der(11)t(11;16)(p11;?), der(11;17)(p10;q10),+13,der(16),t(11;16)(q12;q12)x2,+16,-18[2] 46,XX[4].	51%	11q23(MLLx3) 48% 13q14(RB1x3) 31% 17p13(P53x1) 35% 21q22(AML1x2) 99%	200	1.4	9%	10%	5%	10%
28	5-AZA, CER	RAEB-2	ND	ND	ND	146	0.5	13	ND	ND	ND
31	5-AZA	AML	ND	ND	ND	271	0.2	27	ND	ND	ND

**Δt, time in months from start of treatment with lenalidomide. †RBC, red blood cell; LEN, lenalidomide; CER, complete erythroid response.[10] ‡WHO category: 5q-, 5q- syndrome; RAEB, refractory anemia with excess blasts; AML, acute myeloid leukemia. §FISH 5q, proportion of cells with 5q-deletion as determined by FISH for 5q31(EGR1). ND: not done.*

a gain of the *MLL*- and the *RB1*-loci could be confirmed (Table 1, Figure 1). In December 2007 she was started on 5-azacytidine and became transfusion independent, although there was no decrease in bone marrow blasts. After 8 cycles she progressed to full leukemia and succumbed to an infection.

Cell culture and gene expression

Pathway analysis based on the gene expression profiles of cultured cells (86-96% del[5q] by FISH) demonstrated altered apoptosis and integrin signaling at treatment failure compared pre-treatment, which may reflect a more aggressive disease and an altered interaction between MDS progenitors and stroma.

We also assessed the effect of lenalidomide on mRNA expression of *SPARC* and *Activin-A*, based on data from a previous study.[7] Both genes were up-regulated in post-treatment samples (3.6-7.1 and 2.5-5.1 fold, respectively), similarly to our findings in lenalidomide naïve samples.[7]

As the complex karyotype at progression included trisomy 13 we assessed the expression level for the *FLT3* gene, located at 13q12. There was an upregulation of *FLT3* at the time of progression, however, from a low pre-treatment level.

Mutated TP53 pre-treatment

Before treatment with lenalidomide, 1-2% of the BM progenitors over-expressed p53, which may indicate a mutation in the *TP53* tumor suppressor gene.[11] *TP53* plays a crucial role in genomic integrity and stability.[12] Interestingly, this subclone expanded in parallel with the blast count at disease progression (Table 1, Figure 1). *TP53* was sequenced in pre- and post-treatment samples. Before treatment, an A>G mutation in exon 5 (Y163C) was identified at a similar levels as the wild-type gene. At time of progression the mutated gene dominated, consistent with the cytogenetics demonstrating a complex karyotype including del(17p13) resulting in loss of the one *TP53* allele (Table 1).

TP53 mutations are exceedingly rare in the 5q- syndrome,[13] however, they frequently occur in MDS with

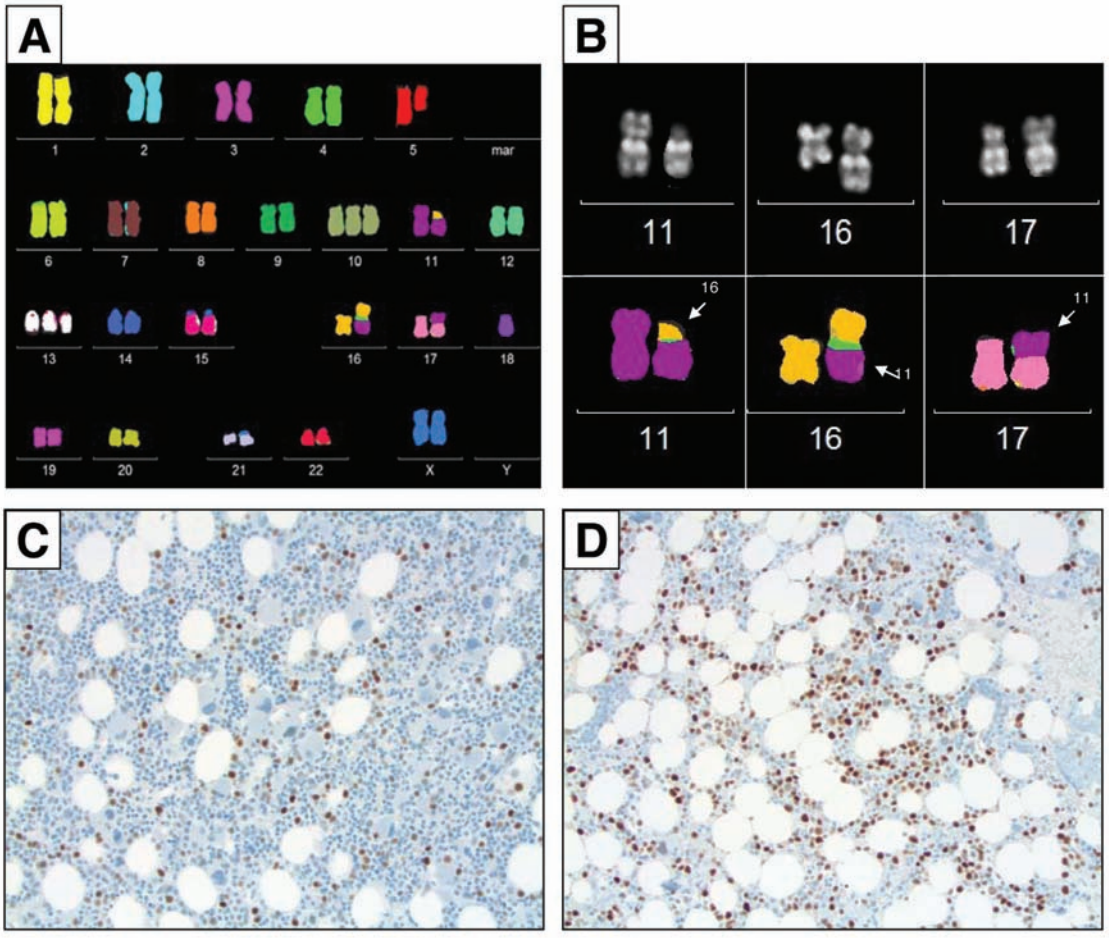

Figure 1. (A) Karyogram of a metaphase at time of progression shown by M-FISH. (B) Partial karyograms of the structural aberrant chromosomes 11, 16 and 17 shown in detail. After M-FISH the additional chromosomal material in the short arm of the derivative chromosome 11 could be identified as material of chromosome 16: der(11)t(11;16)(p11;?). The additional chromosomal material in the long arm of the derivative chromosome 16 and the additional material in the short arm of the derivative chromosome 17 were identified as material of chromosome 11: der(16)t(11;16)(q12;q12) and der(11;17)(p10;q10). Immunohistochemistry demonstrated (C) a population aberrantly over-expressing p53 already pre-treatment, and (D) a clear increase of this population at disease progression.

complex karyotypes including del(5q) or in therapy-related MDS, invariably implying a poor outcome,[14,15] The frequency of small fractions of BM progenitors with abnormal p53 expression, which may fall below the detection threshold for a mutational analysis, is unknown in MDS with isolated del(5q).

Potential prognostic implications of p53 expressing subclones pre-treatment

The *TP53* mutated clone in our case was present before treatment with lenalidomide, remained stable despite a partial cytogenetic response, and expanded at progression. We argue that this clone consisted of cells with inherent genomic instability which increased their probability of acquiring molecular features making them less sensitive to lenalidomide. The p53 expressing clone expanded in parallel to the blast count at time of transformation, and the complex karyotype evolving included deletion of the *TP53* locus at 17p13 in addition to trisomy 13, incorporating the *FLT3* gene, and rearrangements leading to a gain of the *MLL* locus on chromosome 11. Consequently, inactivation of p53 and acquisition of additional genetic aberrations are likely to have contributed to progressive disease in this patient. Moreover, the presence of trisomy 13 at time of disease progression during lenalidomide treatment is in contrast to a recent report suggesting that AML patients with trisomy 13 may respond particularly well to lenalidomide.[16]

Leukemic evolution occurs in around 10% of patients with classical 5q- syndrome (blasts <5% and an isolated del[5q]) during the natural course of the disease, while the risk of progression is higher in the presence of additional cytogenetic aberrations or a blast increase.[17] The cytogenetic characteristics at time of transformation have not been well studied. A preliminary report indicated that 7 of 22 (32%) MDS patients with less than 10% BM blasts and a karyotype including del(5q) receiving lenalidomide developed complex karyotypes at time of progression, at a median of 44 months from diagnosis.[18] Of the 7 patients who progressed, 2 patients had a true low-risk 5q- syndrome, while 5 had a blast count 5-9% or del(5q) with one additional chromosomal abnormality. In addition, a recent report described a patient with classical low-risk 5q- syndrome that transformed to AML with a highly complex karyotype after eight months of treatment with lenalidomide.[19] The European Medicines Agency (EMEA) recently advised against the approval of lenalidomide in Europe for patients with low-risk MDS and del(5q) stating that a treatment-associated increase in the risk of leukemic transformation could not be excluded.[20]

It is conceivable that lenalidomide may suppress a favorable clone and allow expansion of a more malignant subclone; however, it is equally conceivable that the potent effect of lenalidomide may delay progression. This question is best addressed in a randomized trial. A first step could be to analyze the association of p53 overexpression and *TP53* mutation with the probability of disease progression in an expanded cohort of MDS del(5q) patients. This will not answer the question whether lenalidomide affects the natural course of the disease; however, it may identify a subgroup of patients at higher risk of transformation who should be carefully monitored and in which other treatment modalities should be explored. The presence of easily detectable subclones with inactivated p53, and thereby a more malignant potential, represents a novel concept in low-risk MDS and may have important prognostic implications.

Authorship and Disclosures

Designed research: MJ, LS, GG, APo, BS, EHL; managed the patient: MJ and EH-L; generated and analyzed data: MJ, LS, AP, GG, JSW, JB, APo, BS, EH-L; wrote the paper: MJ, LS, GG, APo, BS, EH-L.

MJ received lecture fees from Celgene and is co-investigator of Celgene MDS-004 trial. BS and GG are part of the central reference laboratory for cytogenetics in the Celgene MDS-004 trial. EHL is the Swedish principal investigator in the Celgene MDS-004 trial.

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