Genome-wide analysis of copy number changes and loss of heterozygosity in myelodysplastic syndrome with del(5q) using high-density single nucleotide polymorphism arrays

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ABSTRACT

Background
We undertook a genome wide single nucleotide polymorphism analysis of a spectrum of patients with myelodysplastic syndrome del(5q) in order to investigate whether additional genomic abnormalities occur. Single nucleotide polymorphism array analysis has been shown to detect not only gene deletions but also regions of uniparental disomy that can pinpoint particular regions for mutation analysis.

Design and Methods
We studied 42 cases of myelodysplastic syndrome with del(5q), comprising 21 patients with 5q- syndrome and 21 with del(5q) (not 5q- syndrome), and 45 healthy controls by genome wide single nucleotide polymorphism analysis with the 50K Affymetrix single nucleotide polymorphism arrays.

Results
The del(5q) was characterized in all cases. The commonly deleted region of the 5q- syndrome extends between the genes *SH3TC2* (proximal boundary) and *GLRA1* (distal boundary) and measures 2.9 Mb. Copy number changes in addition to the del(5q) were observed in 10 of 21 patients with del(5q) myelodysplastic syndrome but in none of the patients with the 5q- syndrome. A total of 63 regions of uniparental disomy greater than 2 Mb were detected in 40 of 42 patients, dispersed on 18/23 chromosomes. In the 5q- syndrome group 31 regions of uniparental disomy were identified in 19 of 21 patients, the largest one being 7.6 Mb. All 21 patients with del(5q) myelodysplastic syndrome had uniparental disomy; in total 32 regions of uniparental disomy were identified in the 21 patients, including six regions of uniparental disomy > 10 Mb. Eight recurrent regions of uniparental disomy were observed among the 42 patients. For eight patients we had T-cell DNA as a germline control and four recurrent regions of uniparental disomy were identified that were present only in the neutrophil and not T-cell DNA. One small region of uniparental disomy at 10p12.31-p12.2 was observed in four patients with the 5q- syndrome.

Conclusions
This study shows that regions of uniparental disomy greater than 2 Mb are found in the 5q- syndrome and del(5q) myelodysplastic syndrome, although large regions of uniparental disomy (>10 Mb) are only found in the latter group. The recurrent regions of uniparental disomy may indicate the position of novel leukemia-associated genes.

Key words: uniparental disomy, 5q- syndrome, del(5q) myelodysplastic syndromes.

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The online version of this article contains a supplementary appendix.

Introduction

The myelodysplastic syndromes (MDS) are a heterogeneous group of clonal disorders of the hematopoietic stem cell characterized by ineffective hematopoiesis,[1] with approximately 30% to 40% of cases transforming to acute myeloid leukemia (AML).[2,3] An interstitial deletion of the long arm of chromosome 5, del(5q), is the most common chromosomal abnormality in MDS with a frequency of 10-15% among all patients.[4] del(5q) also occurs in AML[4] and several other cancers.[5-7] The 5q- syndrome is defined as an MDS with a bone marrow blast count <5% and the presence of the del(5q) as the sole chromosomal abnormality.[8,9] The 5q- syndrome has a good prognosis and a low probability of transformation to AML.[10,11] However, in high-risk MDS and *de novo* AML a del(5q) is associated with a poor prognosis.[12]

Single nucleotide polymorphism (SNP) microarrays allow the simultaneous analysis of large numbers of polymorphic loci across the genome, which provides information on copy number change and/or loss of heterozygosity (LOH),[13,14] LOH can result from deletion of all copies of one homolog or from uniparental disomy (UPD) in which both copies of a chromosome pair have the same parental origin. UPD can arise through a number of mechanisms including incomplete segregation of chromosomes and mitotic recombination. Conventional cytogenetic methods do not detect UPD, whereas UPD can be efficiently detected by SNP microarrays.[15,16] UPD has been described in many cancers, including AML, in which an association between UPD and homozygous gene mutation was found. In seven of 13 cases of AML with UPD, homozygous mutations at four distinct loci were found in the regions affected by UPD.[17] A recent study showed that UPD is a prominent feature of some diseases such as chronic lymphocytic leukemia and myeloma.[18,19]

In this study we used SNP arrays to identify cryptic copy number changes and regions of UPD in patients with MDS and a del(5q), including patients with the 5q- syndrome.

Design and Methods

Patients and samples

Forty-two patients with MDS and 45 healthy volunteers were included in this study. The MDS patients were classified according to French-American-British (FAB) criteria. All MDS patients had a deletion of the long arm of chromosome 5 [del(5q)], and 21 patients had the 5q- syndrome. The samples from MDS patients were obtained from several sources: Oxford and Bournemouth (United Kingdom), Duisburg (Germany), Stockholm (Sweden) and Pavia (Italy). The study was approved by the ethics committees of the institutes involved (Oxford C00.196, Bournemouth 9991/03/E, Duisburg 2283/03, Stockholm 410/03, Pavia 26264/2002) and informed consent was obtained. The patients' characteristics are shown in Table 1.

DNA preparation

Peripheral blood neutrophils were isolated using Histopaque (Sigma-Aldrich) and pelleted after hypotonic lysis of erythrocytes and two washes in phosphate-buffered saline. The purity of the neutrophil populations was high, >95%, as assessed by standard morphology on Wright-Giemsa-stained cytospin preparations. For eight MDS patients, T cells were isolated from peripheral blood by MACS with $CD3^+$ microbeads, for paired sample analysis. Genomic DNA was isolated by phenol-chloroform extraction using standard methods and evaluated for quality and concentration using an ND-1000 spectrophotometer (Nanodrop, Wilmington, USA).

SNP mapping assay

The SNP mapping assay was performed according to the protocol supplied by the manufacturer (Affymetrix, Santa Clara, CA, USA). Briefly, 250 ng DNA were digested with Hind III, ligated to the adaptor, and amplified by polymerase chain reaction (PCR) using a single primer. PCR products were purified with the DNA amplification clean-up kit (Clontech) and the amplicons were quantified. The 40 μg of purified amplicons were fragmented, end-labeled and hybridized to a Genechip Mapping 50K *Hind* III array at 48°C for 16-18 hours in a Hybridization Oven 640 (Affymetrix). After washing and staining in a Fluidics Station 450 (Affymetrix), the arrays were scanned with a GeneChip Scanner 3000 (Affymetrix).

SNP array data analysis

Cell intensity calculations and scaling were performed using GeneChip Operating Software (GCOS). Data were analyzed using GeneChip Genotyping Analysis Software Version 4.0 (Affymetrix) and CNAG software version 2.0. Quality control was performed within the Genotyping software after scaling the signal intensities of all arrays to a target of 100%. DNA copy number was analyzed with both the chromosome copy number tool (CNAT) version 3.0 and CNAG version 2.0. CNAT compares obtained SNP hybridization signal intensities with SNP intensity distributions of a reference set from more than 100 healthy individuals of different ethnicity. For analysis with CNAG we used the pool of 45 healthy controls included in the study as a reference set.

Table 1. Patients' characteristics.

	Data
Median age, years (range)	69, (24-88)
N. of men/n. of women	9/33
FAB subtype	
Refractory anemia	29
Refractory anemia with ringed sideroblasts	2
Refractory anemia with excess blasts	9
AML, chronic myelomonocytic leukemia	2
5q syndrome	21
del(5q) MDS	21

Mutation analysis

Coding exons of *SPAG-6* were amplified from the neutrophil DNA of 12 patients with del(5q) MDS, including ten patients with the 5q- syndrome, by PCR. Primers and amplifications conditions are available on request. PCR products were purified using ExoSAP-IT (GE Healthcare) and directly sequenced using a BigDye Terminator v1.1 cycle sequencing kit and an ABI 3100 Genetic analyzer. Sequence data were analyzed using Sequence Analysis 1.6.0 (Informagen).

Results

Quality control of SNP genotype arrays

The accuracy of whole-genome sampling analysis was determined by the SNP call rate generated via GCOS software. The mean genotyping call rates were 96.72% for the patients' samples and 95.40% for the control samples. By calculation of heterozygous SNP calls within the X chromosome in 28 males, the accuracy of the SNP array was estimated to be 99.68%.

Del(5q) confirmed and 5q- syndrome commonly deleted region defined

DNA copy number at each SNP locus is calculated from the corresponding signal intensity on the array. The del(5q) was observed in all 42 patients included in the study; the extent and size of the deletions are given in *Online Supplementary Table S1*. The boundaries of the commonly deleted region in the 21 patients with the 5q- syndrome were between the genes *SH3TC2* at the proximal boundary (physical position 148362828 bp) and *GLRA1* at the distal boundary (physical position

Table 2. del(5q) MDS patients with additional karyotypic abnormalities as determined by cytogenetic analysis, and copy number change identified by SNP array. Those abnormalities detected by only one of the methodologies are highlighted in bold in the respective column.

Patient n.	*FAB subtype*	*Cytogenetic karyotype*	*Copy number change by SNP array*	
			Copy number loss	*Copy number gain*
7	RAEB	43-45,XY,del(5)(q31),**der(7),t(7;12)(q22;q1?3)**,-12,-13,**-19**,?del(20)(q1?3) [cp4]	5q21.1-q35.3, **7q11.22-q36.3**, 12p12.1-p13.2, 13q14.11-q14.2, 13q14.2-q21.1, **15q12-q13.2, 17p11.2-p13.3**, 20q11.21-q13.13	
16	CMML	46,XY,del(5)(q13q33),del(13)(q12q22) [20]	5q14.3-q33.3, 13q13.2-q21.31	
17	RA	46,XX,del(5)(q13q33),**t(5;13)(q31;q14),t(12;17)(q22;p11),inv(17)(q11p11)**	5q14.3-q33.3, **12q21.31-q22, 12p13.2-p13.1**	
23	RAEB	44,XX,del(5)(q14q33),-7,-13,-14,-18,-20,+mar1,+mar2,+mar3[9]/46,XX[1]	**4q21.1- q35.2**, 5q21.1-q33.2, 7p22.3- q36.3, **9q21.11- q34.3**, 13q21.1-q21.31, 13q14.13-q14.3, 14q24.2-q24.3, **17p13.3-p11.2**, 20p13- p12.1	
24	RA	Not known	2p23.3, 5q22.1-q33.2	Whole chromosome 8
25	RAEB	46,XX,del(5)(q13q33),**t(6;12)(q13p12)**[2]/ 45,XX,del(5)(q13q33),**t(6;12)(q13p12)**,-7**,-22**,+mar [15]/46,XX[3]	5q14.2-q34, 7p22.3-p11.2, 7q21.3-q36.3	
40	RAEB	45,XX,**-2**,del(5)(5pter-5q23),**-11,der(11)(11pter-11q12::?2p21-2pter)[4]/45,XX,del(2)(2qter-2p21)**, del(5)(5pter-5q23)**,-11,der(11)(11pter11q12::?2p21-2pter)**,**-20**[5]/46,XX[4]	5q23.1-q34	
42	RA	46,XX,del(5)(q?13q?33),del(11)(q?22)[3]/46,XX[2]	5q14.3-q34, 11q22.3- q25	
26	RAEB	92,XXYY,del(5)(q14q33),del(5)(q14q33)[8]	5q21.3-q35.3	**13q31.2-q34**
18	RA	46,XX,del(5)(q13q33)[5]/47,XX,del(5)(q13q33),+8[22]/46,XX [2]	5q14.3-q33.3	Whole chromosome 8
35	RA	46,Y,der(X)**t(X;12)(p22q21)**,del(5)(q14-15q33-34),der(12)del(12)(p11q13)[7]/46,XY[3]	5q15-q33.3,**6q23.2-q23.3**, 12p11.23-p13.31, **12q21.33-q22**	
36	RA	46,XX,del(5)(q14q34),**t(1;3)(q33;p14)**[21]/46,XX[4]	5q14.3-q33.2	
30	RA	46,XX,del(5)(q13q33),**del(11)(q23)**	5q23.1-q33.2	
15	AML	46,XX,9q+[1]/46,XX,del(5)(q31q33),**9q+** [19]	5q14.2-q33.3	

When a monosomy was recorded by cytogenetics and a deletion by SNP array we classed them as the same abnormality. All other patients have del(5q) only as determined by both cytogenetics and SNP array and the extent of the del(5q) for every patient is given in Online Supplementary Table S1. RA: refractory anemia; RAEB: RA with excess blasts; CMML: chronic myelomonocytic leukemia.

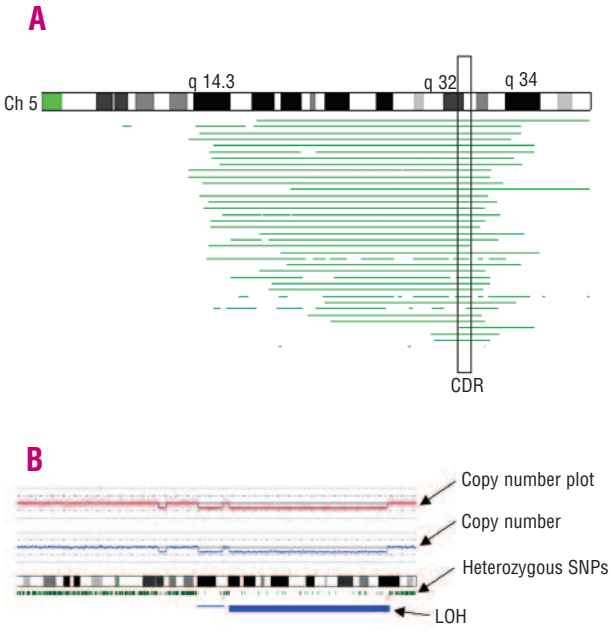

Figure 1. (A) Integral view of chromosome 5 in all patients with the 5q- syndrome and del(5q) MDS. Deletion of 5q was detected in all samples. Green lines under the chromosome indicate the deleted regions in each case. The rectangle shows the commonly deleted region (CDR). (B) Non-contiguous 5q deletion. In the upper histogram the red dots represent the log2 intensity ratio for each SNP locus and the blue line below it shows the averaged log2 values. The corresponding chromosome ideogram and location of heterozygous SNP calls (small green vertical bars) are shown. The blue bar below the ideogram indicates regions of LOH.

151297473 bp) (Figure 1A). Using SNP array technology the commonly deleted region measures approximately 2.9 Mb.This confirms our previous mapping data of the 5q- syndrome commonly deleted region,[20] provides more precise definition of the region's boundaries and a more accurate estimation of its size. When taking all 42 patients into consideration the commonly deleted region remained unchanged.

In one patient with the 5q- syndrome the del(5q) was shown to be non-contiguous, (Table 2 and Figure 1B), an abnormality not revealed by conventional cytogenetic methods.

Detection of additional copy number changes in myelodysplastic syndromes patients with del(5q)

None of the 21 patients with the 5q- syndrome had copy number changes in addition to the del(5q). Copy number changes in addition to the del(5q) were observed in ten of the other 21 MDS patients. In five of these patients the copy number changes were not identified by cytogenetics (Table 2). For example, patient #26 was shown to have amplification of 13q31.2-q34 by SNP array (Table 2). Nine patients had abnormalities documented by cytogenetics that were not seen by SNP array, the majority of these abnormalities were translocations or inversions.

In three patients with chromosome translocations (see Table 2, patients #7, 17 and 35), SNP array analysis identified a deletion that encompassed the translocation breakpoint defined by karyotyping.

Regions of LOH with normal copy number - UPD

Regions of UPD were defined as continuous stretches of homozygous SNP calls >2 Mb without copy number loss.[21] In this study a total of 90 regions of UPD >2 Mb, dispersed across 17 chromosomes were identified in 36/45 normal control subjects. The majority of these regions were between 2 Mb and 3 Mb.

For the patients we report only those regions of UPD that were not observed in any of the normal healthy controls included in our study or in normal healthy control subjects reported in the literature.[21,22] A total of 63 regions of UPD > 2 Mb, dispersed on 18/23 chromosomes, were detected in 40 of 42 patients (*Online Supplementary Table S2*) (Figure 2a). In the 5q- syndrome group, 31 regions of UPD > 2 Mb were identified in 19 of 21 patients, the largest one being 7.6 Mb. Thirty-two regions of UPD > 2 Mb were identified among all 21 del(5q) MDS patients, including six regions of UPD >10 Mb (Figure 2b). In the 5q- syndrome group the frequency of UPD regions was highest on chromosome 4 and in the del(5q) MDS group on chromosomes 6 and 13 (Figure 2A).

Recurrent regions of UPD

We identified eight regions of UPD > 2 Mb in two or more patients and in none of the controls; 4q21.21 (two patients), 4q13.1 (three patients), 4q26-q27 (two patients), 4q13.1- q13.2 (two patients), 6q13- q14.1 (four patients), 6q14.3-q15 (two patients); 8q21.11 (two patients), 9p21.1 (two patients). The sizes of these recurrent regions of UPD and the genes that map within them are shown in Table 3.

Paired sample analysis

For eight patients in the study we obtained DNA from their T cells as germline control material. For this paired sample analysis we recorded all regions of UPD >1 Mb that were present in the neutrophil, but not T-cell sample of each patient. In total we identified 50 regions of UPD > 1 Mb. Four regions of UPD > 1 Mb were identified in two or more patients: 3q25.32 (two patients), 5p14.3-p14.2 (two patients), 6q12 (two patients) and 10p12.31-p12.2 (four patients).

The four patients with the recurrent UPD region at 10p12.31-p12.2 were all classified as having 5q- syndrome. Seven genes map to this region; *SPAG6*, *MLLT10*, *PIP5K2A*, *BM1*, *COMMD3*, *NEBL* and *DNAJC1*.

Candidate gene *SPAG6* mapping to this recurrent region of UPD at 10p12.31-p12.2 was analyzed for the presence of mutations. All 11 exons of *SPAG6* were sequenced in ten patients with the 5q- syndrome, including the four patients with the corresponding UPD, and two patients with del(5q) MDS. No mutations were identified.

In addition, two regions of LOH with associated deletions at 12p13.2-p13.1 and 12q21.31-q22 were detected in the neutrophil but not T-cell sample of a patient with del(5q) MDS.

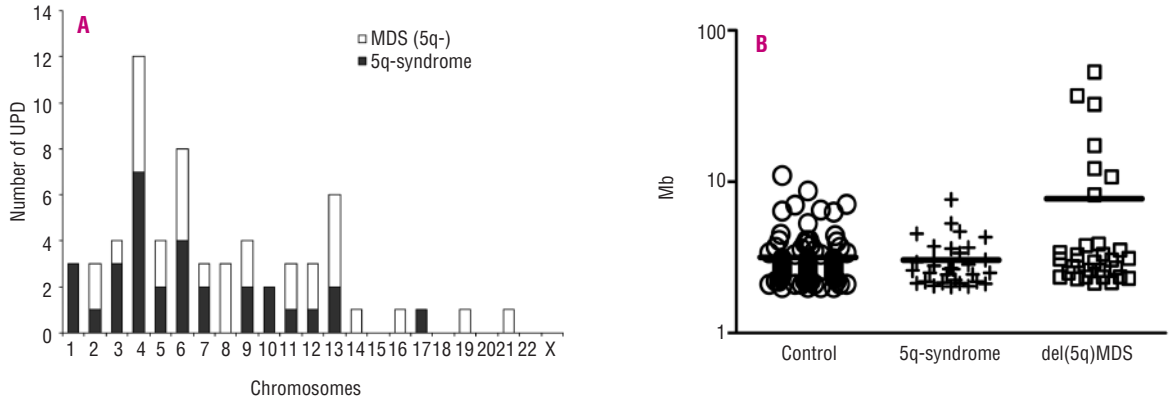

Figure 2. UPD in 5q- syndrome and del(5q) myelodysplastic syndromes. (A) Frequency of UPD on each chromosome. Each bar represents the two patient groups: the top one (white) represents the number of UPD in del(5q) MDS (not 5q- syndrome), the bottom one (black) the number of UPD in 5q- syndrome. No UPD were found on chromosomes 15, 18, 20, 22, and X. (B) UPD size distribution in normal controls, patients with 5q-syndrome and patients with del(5q) myelodysplastic syndromes. The bold black bars show the mean size of the UPD.

Table 3. Recurrent regions of UPD identified in patients with the 5q- syndrome and del(5q) myelodysplastic syndromes.

	Recurrent ID		Physical position		Length	
Cytoband	*N. of cases (%)*	*Patient N.*	*From*	*To*	*(Mb)*	*Candidate genes*
4q13.1	3 (7%)	39/2/23	60577199	61810493	1.23	None
4q26-q27	2 (4.6%)	13/20	120601325	121421102	0.82	*PDE5A, MAD2L1, SAR1P3*
4q13.1- q13.2	2 (4.6%)	20/23	65578799	66861924	1.28	*EPHA5, XR_018421.1*
4q21.21	2 (4.6%)	1/34	80195990	82530576	2.33	*ARD1B, GK2, ANTXR2, PRDM8, FGF5, C4orf22, BMP3, PRKG2*
6 q13- q14.1	4 (9.3%)	31/41/21/34	75035581	76259717	1.22	*COL12A1, COX7A2, TMEM30A, FILIP1*
6 q14.3-q15	2 (4.6%)	31/12	85411634	88478204	3.06	*TBX18, NT5E, SNHG5, SNX14, SYNCRIP, Q5T5W8_HUMAN, HTR1E, CGA, ZNF292, C6orf162, RARSL, GJB7, Q5TEZ4_HUMAN, Q5TEZ5_HUMAN, C6orf165, ORC3L*
8q21.11	2 (4.6%)	16/32	75982355	78287079	2.30	*CRISPLD1, HNF4G, ZFHX4, PXMP3*
9p21.1	2 (4.6%)	31/37	28134832	30212869	2.07	*LINGO2*

Discussion

Using SNP array analysis, we analyzed a group of 42 patients with MDS and del(5q) for the presence of copy number changes and LOH. Twenty-one patients with the 5q- syndrome were compared with 21 patients with del(5q) MDS. Patients with the 5q- syndrome have a good prognosis and this may be related to their relative genetic stability as evidenced by an absence of other cytogenetic abnormalities and a failure to date to find other leukemia-associated mutations in this group of patients in contrast to other MDS patients with the del(5q).[23]

Nevertheless hitherto unidentified genetic abnormalities in addition to the del(5q) may exist in the 5q- syndrome. The high density SNP arrays offer a very good method of analysis for determining genomic abnormalities which may have been missed by earlier technologies. SNP analysis also provides for an accurate, efficient comparative method of examining large genomic deletions - giving us the opportunity for a new analysis of the critical deleted region in the 5q- syndrome, which has confirmed and refined our previous analysis.

Additional copy number changes were detected in 10/21 del(5q) MDS patients. In general the karyotypic abnormalities detected by cytogenetic analysis were confirmed by SNP array analysis. Some additional abnormalities were detected by SNP array and the greater resolution achieved by this method provided a more accurate definition of abnormalities, particularly in cases with a complex karyotype. A proportion of lesions detected by cytogenetic analysis were not found by SNP array. A limitation of SNP arrays is the inability to distinguish multiple clones, and minor clones may be detected by cytogenetics but not by SNP array analysis. In this study, the majority of lesions missed by the SNP array were translocations; this dis-

crepancy is not unexpected as SNP array technology is unable to detect balanced rearrangements. Interestingly, cryptic deletions were detected at the location of translocation breakpoints in three cases. Such cryptic deletions associated with *balanced* translocations have been reported previously.[24] For example, a deletion associated with the 13q14 translocation in B-cell leukemia pin-pointed a region of genetic importance in this disorder.[25]

In marked contrast to del(5q) MDS patients none of the 5q- syndrome patients showed any additional copy number changes. Again, as with the mutation study referred to above,[23] this result is consistent with the good prognosis of the 5q- syndrome.

In this study, a total of 90 regions of UPD, dispersed across the genome on 17/23 chromosomes, were identified in 36 control subjects. The majority of the regions of UPD were between 2 and 3 Mb in length. The frequency of regions of UPD in normal individuals observed in this study is comparable with that observed by Gibson *et al.*, who examined the genome-wide SNP genotypic data from the 209 unrelated individuals from the four HapMap populations. They observed 1393 tracts of consecutive homozygous SNP exceeding 1 Mb in length among the 209 individuals.[22] These studies show homozygous tracts are common in normal individuals and that a control pool is essential for the analysis of SNP array data.

SNP analysis that enables the detection of UPD has already proven very informative in the study of hematologic malignancies. An illustrative example is the UPD seen in some cases of polycythemia vera that corresponds to the location of the *JAK2* gene on chromosome 9p.[26,27] By analogy with polycythemia vera, it is considered that SNP analysis may give a clue to the location of disease genes in MDS, as some studies have already confirmed in AML.[15,17]

For the patient group as a whole we determined disease-related UPD on the basis of size (>2 Mb) and also the absence of the corresponding UPD in control groups. We found that regions of UPD were very common in the patients although the del(5q) MDS group clearly showed a greater number of large UPD regions than did the 5q- syndrome group. There was no correlation between the number of copy number changes and number of regions of UPD per patient. However, the majority of large regions of UPD (>10 Mb) were in patients who also had regions of copy number change, suggesting a common underlying genetic instability. The regions of UPD are not evenly distributed across the various chromosomes, since their frequencies are higher on chromosomes 4, 6 and 13. Eight regions of UPD were observed in two or more patients: four different regions on 4q, two different regions on 6q, one region on 8q and one on 9p. A recent study of low-risk MDS by SNP array analysis also highlighted chromosome 4 as having the highest number of regions of UPD.[21] Twelve regions of UPD ranging from 2.6-138.5 Mb were identified on chromosome 4 by Mohamedali *et al.*, some of which overlap with the regions identified in our study.

For the subset of patients for whom we had corresponding T-cell DNA for direct intrapatient comparisons we were able to lower the stringency of the UPD definition to regions greater than 1 Mb. In this analysis we identified four recurrent regions of UPD. Four patients with the 5q- syndrome had a region of UPD at 10p12.2-10p12.3. identified only in the neutrophil but not T-cell samples. Seven genes mapping to this recurrent region of UPD, *SPAG6, MLLT10, PIP5K2A, BM1, CommD3*, *NEBL* and *DNAJG1*, are now to be considered as potential candidate genes for mutation in the 5q- syndrome. Gene expression profiling studies identified *SPAG-6* as one of the most significantly upregulated genes in the $CD34^+$ cells from patients with the 5q- syndrome when compared with both normal individuals and patients with refractory anemia and a normal karyotype.[28] In addition, *SPAG-6* has recently been shown to be markedly overexpressed in pediatric AML,[29] hence this gene was a good candidate to search for mutations which would be rendered homozygous by UPD. We analyzed the 11 exons of *SPAG-6* for the presence of mutations in the four 5q- syndrome patients with chromosome 10p12.2-10p12.3 UPD, six additional patients with the 5q- syndrome and two patients with del(5q) MDS. No mutations were identified, although further investigation of *SPAG6* in MDS is warranted.

In conclusion, we have identified recurrent regions of UPD in patients with MDS which may be of pathogenetic importance. In addition we have shown a clear distinction between 5q- syndrome and other del(5q) MDS cases with regards to both copy number changes and the presence of large UPD, consistent with the different clinical behavior of patients with these two disorders.

Authorship and Disclosures

CF, JSW and JB designed the research; LW, CF, AG, MC, LM, SK and CA performed the research; LW and CF analyzed the data; LW, CF, JSW and JB wrote the paper. The authors reported no potential conflicts of interest.

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