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Frequent mutation of the polycomb-associated gene *ASXL1* in the myelodysplastic syndromes and in acute myeloid leukemia

The identification of those genes that are frequently mutated in malignancies is essential for a full understanding of the molecular pathogenesis of these disorders, and often for the provision of markers for the study of disease progression. Recently, mutation of the *ASXL1* (additional sex combs 1) gene has been reported in 4 out of 35 patients (11%) with myelodysplastic syndromes (MDS) and in 17 out of 39 patients (43%) with chronic myelomonocytic leukemia (CMML), a disease classified as MDS/myeloproliferative disorder.[1] The mutations were all found in exon 12 of the gene and are predicted to lead to the truncation of the C-terminus of the protein, which contains a PHD finger.[1] The PHD finger is a structural motif found in nuclear proteins involved in chromatin modifications. The function of the human ASXL1 protein is poorly understood, but there is some evidence to suggest that it may represent a component of DNA- and/or histone-modifying complexes.[2] *ASXL1* belongs to a family of three members that encode polycomb and mixed-lineage leukemia/trithorax chromatin modifier complexes.[2] Most recently, mutations of *ASXL1* have been reported in 5 out of 64 (approximately 8%) patients with the myeloproliferative disorders essential thrombocythemia and primary myelofibrosis, and in 11 out of 63 (17%) patients with acute myeloid leukemia (AML).[3,4] These data suggest that *ASXL1* represents a new tumor suppressor gene, the inactivation of which may have an important role in the molecular pathogenesis of malignant myeloid disorders.

We have screened *ASXL1* for mutations in a large group of patients with MDS, CMML and AML, in order to determine their frequency in these malignant myeloid disorders and to determine any association with specific MDS subtypes, karyotypic groups and disease progression. Moreover, we have determined the effect of these mutations on global gene expression patterns and gene pathways.

A total of 300 patients with MDS, AML or CMML and 111 healthy controls were included in this study. 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, Pamplona—approval by the Investigational Review Boards at the University of Navarra) and informed consent was obtained. Genomic DNA was isolated from patient bone marrow or peripheral blood samples by phenol/chloroform extraction using standard methods and evaluated for quality and concentration using an ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). All previously reported mutations of *ASXL1* in hematological malignancies were found exclusively in exon 12 of the gene.[1,3]

Sequences spanning exon 12 of the *ASXL1* gene were amplified by PCR from the DNA of 300 patient samples and 111 normal controls. Primers and amplification conditions were as previously published.[1] PCR products were purified and directly sequenced using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3100 Genetic analyzer. Sequence data were analyzed using Mutation Surveyor V3.25 (Softgenetics, State College, PA, USA).

ASXL1 mutations were observed in 62 out of the 300 patients (21%; Table 1). *ASXL1* mutations were found in 5 out of 79 MDS patients with refractory anemia (RA; 6%), in 17 out of 55 patients with either refractory anemia with excess blasts 1 (RAEB-1) or RAEB-2 (31%), and in 17 out of 67 patients with AML (25%; Table 1). Thus, the mutation frequency is relatively high in late MDS and AML, with a lower frequency across the spectrum of early MDS (Table 1). Examining the mutation frequency by karyotype shows that the mutation is common in

Table 1 *ASXL1* mutations in our series of patients with malignant myeloid disorders

Disease subtype	Frameshift	Nonsense	Total
RA, 5q- syndrome (n = 36)	1 (3%)	1 (3%)	2 (6%)
RA (n = 43)	2 (5%)	1 (2%)	3 (7%)
RCMD (n = 10)	—	—	0 (0%)
RARS (n = 29)	5 (17%)	—	5 (17%)
RCMD-RS (n = 9)	1 (11%)	—	1 (11%)
RAEB-1 (n = 29)	9 (31%)	—	9 (31%)
RAEB-2 (n = 26)	5 (19%)	3 (12%)	8 (31%)
AML post MDS (n = 40)	7 (18%)	2 (5%)	9 (23%)
AML (n = 27)	8 (30%)	—	8 (30%)
CMML (n = 51)	15 (29%)	2 (4%)	17 (33%)
Karyotypic group			
Normal karyotype (n = 86)	33 (39%)	1 (1%)	34 (40%)
Loss Y (n = 9)	2 (22%)	—	2 (22%)
Del5q/Monosomy 5 (n = 51)	1 (2%)	1 (2%)	2 (4%)
Del 20q (n = 8)	1 (13%)	1 (13%)	2 (25%)
Trisomy 8 (n = 20)	—	2 (10%)	2 (10%)
Del7q/Monosomy 7 (n = 10)	4 (40%)	—	4 (40%)
Other abnormalities (n = 80)	10 (13%)	5 (6%)	11 (19%)

Abbreviations: AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; MDS, myelodysplastic syndromes; RA, refractory anemia; RAEB-1, refractory anemia with excess blasts 1; RAEB-2, refractory anemia with excess blasts 2; RARS, refractory anemia with ringed sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RCMD-RS, refractory cytopenia with multilineage dysplasia and ringed sideroblasts.
The frequency of *ASXL1* mutations is shown in each disease subtype and in different karyotypic groups.
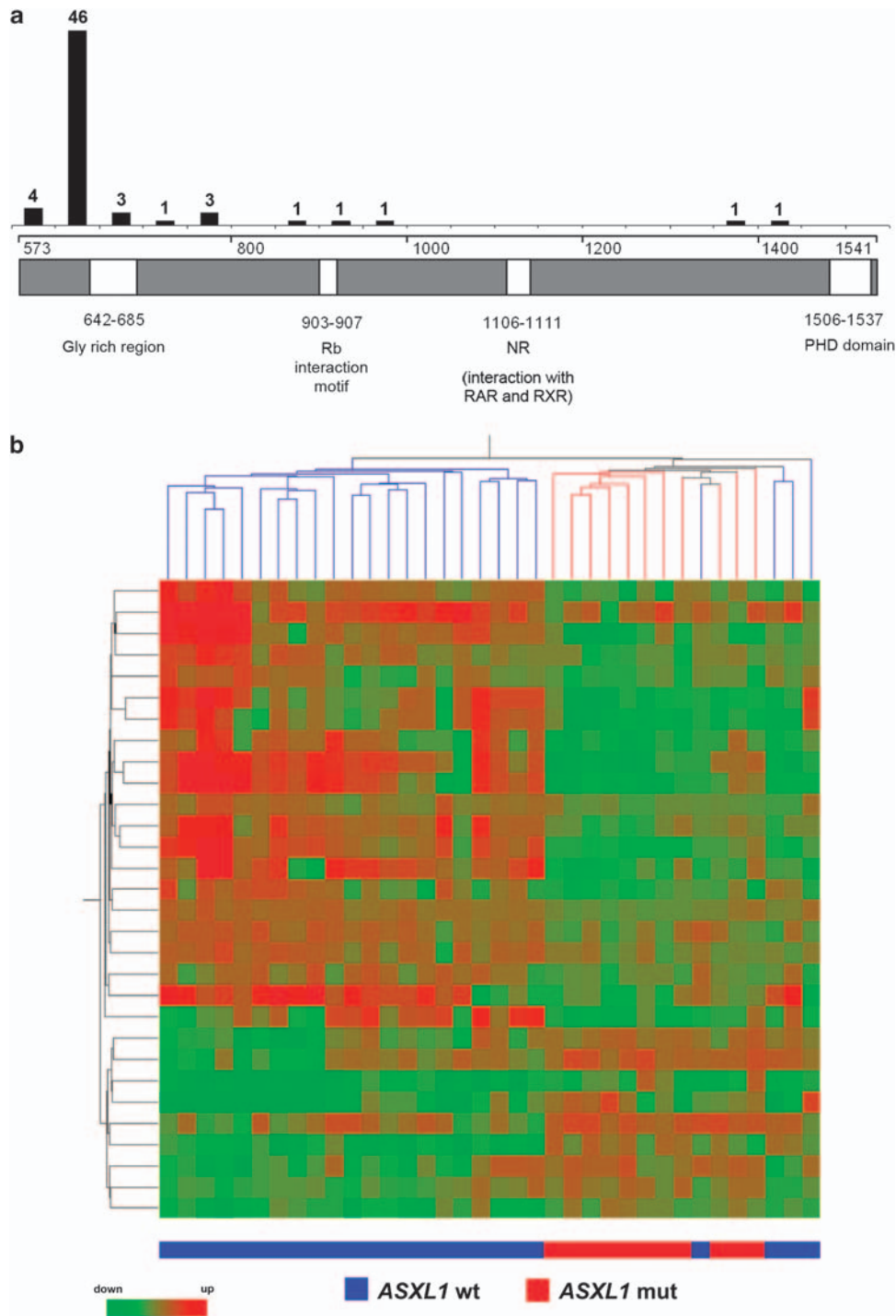
Figure 1 (**a**) *ASXL1* exon 12 functional structure and location of *ASXL1* mutations. The gray bar represents exon 12 of the *ASXL1* gene and the white areas correspond to previously described domains or motifs in the protein. The black bars represent the number of mutations along the exon sequence that lead to a predicted truncated protein. (**b**) Hierarchical clustering of 30 differentially expressed genes between cases with mutation and cases without mutation of *ASXL1*. Each row represents a single Affymetrix probe set and each column a separate patient CD34+ sample (*ASXL1* wild type = blue, *ASXL1* mutated = red).

the normal karyotype group (especially in AML post MDS) and the del(7q)/monosomy 7 group, occurring in 40% of all cases, respectively (Table 1). In contrast, the mutation is uncommon in the del(5q)/monosomy 5 group (Table 1). To investigate whether the mutations were acquired, we analyzed germline DNA from T cells of seven cases. In all cases examined, the *ASXL1* mutations were restricted to the non-lymphoid cells, indicating

that they were acquired. The majority of the mutations identified in this study were heterozygous frameshift mutations caused by deletion or duplication of a nucleotide. The most common mutation was 1934dupG;Gly646TrpfsX12, which accounted for 55% of all mutations. Another three mutations were identified in more than one patient; these were 1900–1922del;E635RfsX15 (8% of mutations), 1872_1894het_del;E630RfsX14 (5%) and

Table 2 Top 10 deregulated pathways in patients with a mutation of *ASXL1* compared with healthy controls

Ingenuity canonical pathway	P-value	Molecules
RAR activation	0.00019	TRIM24, SMAD7, **NFKB2**, **ERCC2**, **SMARCA4**, **CSNK2A2**, ADH7, **PRKCD**, **ADCY1**, **NR2F6**, **NCOR2**, NRIP1, **RXRA**, **RDH13**, **PRKCA**
IL-3 signaling	0.00631	**STAT6**, **PTPN6**, **PAK1**, **PRKCD**, **STAT3**, INPP5D, **PRKCA**
IL-12 signaling and production in macrophages	0.00646	**STAT6**, **IL18**, **PRKCD**, **RAB7A**, **NFKB2**, **RXRA**, **SPI1**, **PRKCA**, DHX9
Primary immunodeficiency signaling	0.00661	**RFXAP**, RAG2, RAG1, **STAT3**, IGHG1, **PDK1**
Semaphorin signaling in neurons	0.00794	CRMP1, **PAK1**, **RHOG**, **FES**, **CFL2**, **RHOU**
GNRH signaling	0.01023	GNAS, **PLCB2**, **PAK1**, **PRKCD**, **ADCY1**, **NFKB2**, MAP3K3, **DNM2**, **PRKCA**
Arginine and proline metabolism	0.01071	**PRODH**, **ALDH3A2**, **OAT**, **ALDH3B1**, **SPR**, **PYCR1**, P4HA2
Valine, leucine and isoleucine degradation	0.01412	ADH7, **ABAT**, **HIBADH**, **ALDH3A2**, **ACAT1**, **ALDH3B1**
Renin–angiotensin signaling	0.01905	**PTPN6**, **PAK1**, **PRKCD**, **ADCY1**, **SHC2**, **STAT3**, **NFKB2**, **PRKCA**
Role of BRCA1 in DNA damage response	0.01950	**E2F6**, RBL1, **FANCA**, **SMARCA4**, RFC3

Genes in regular font are expressed at lower levels in patients with a mutation of *ASXL1* compared with healthy controls, while genes in bold font are expressed at higher levels in patients with a mutation of *ASXL1* compared with healthy controls.

1925het_insA;G643RfsX13 (5%). Indeed, we have found that the majority of mutations are localized to one particular region within exon 12, around the Gly-rich domain (amino acids 642–685) (Figure 1a). Interestingly, 97% of the mutations that lead to a truncated protein are located before the C-terminal nuclear receptor box, which is predicted to interact with the retinoic acid receptor.[5]

None of the previously mentioned mutations were present in the 111 healthy controls. In our control group we found four non-synonymous changes that were not previously reported. In addition to these, we found seven different single base changes in our patient group, five of which were missense changes that could theoretically be polymorphisms and are hence excluded from Table 1.

Therefore, the mutation of *ASXL1* has two features, which should enable it to be used as a molecular marker for studies on minimal residual disease and disease progression. These are (1) its high frequency (and in particular its high frequency in normal karyotype MDS/AML) and (2) the fact that many of the mutations are frameshifts, which provide good targets for sensitive detection assays.

SNP array analysis was performed using Affymetrix Genechip Mapping 50K *Hind*III arrays (Affymetrix, Santa Clara, CA, USA), as previously described,[6] on approximately half (89 of 182) of the MDS patients included in this study, and two patients with mutation of *ASXL1* were shown to have a deletion mapping to 20q encompassing the *ASXL1* gene (data not shown). Intriguingly, the *ASXL1* gene maps close to the critically deleted region identified on 20q in a range of myeloid malignancies.[7]

Gene expression profiling was performed on CD34+ cells obtained from a subset of the MDS patients ($n=29$) and the CMML patients ($n=7$) with and without mutation of *ASXL1*. Gene expression profiling was performed using Affymetrix HG U133 Plus 2.0 arrays as previously described.[8] Affymetrix CEL files were pre-processed using Robust MultiChip Analysis and data analysis was performed using GeneSpring 7.3.1 (Agilent Technologies, Santa Clara, CA, USA). The differentially expressed genes (30 genes, *t*-test $P<0.05$) between the 11 cases with mutation and the 25 cases without mutation of *ASXL1* were used for hierarchical clustering and a good separation between cases with and those without a mutation of *ASXL1* was obtained (Figure 1b), suggesting that patients with mutation of *ASXL1* have a different disease pathophysiology. The Ingenuity Pathway Analysis 7.5 software (Ingenuity Systems, Redwood City, CA, USA) was used to perform pathway analysis on the differentially expressed genes between patients with mutation of

ASXL1 and controls (849 genes, *t*-test $P<0.05$), and this showed that the most significantly deregulated pathway in patients with a mutation of *ASXL1* was the retinoic acid receptor (RAR) activation (Table 2). Several genes in this pathway, including *RXRA*, *PRKCA*, *PRKCD*, *CSNK2A2* and *ADCY1*, showed up-regulation in patients with mutation of *ASXL1*. Intriguingly, ASXL1 has been suggested to function either as a co-activator or as a co-repressor for retinoid receptors RAR and retinoid X receptor (RXR) in a cell type-specific manner.[5,9] The RAR activation pathway was not among the top 50 deregulated pathways in patients without a mutation of *ASXL1*.

The high frequency of *ASXL1* mutations in CMML/MDS/AML makes it a very significant mutation. Moreover, our study shows that *ASXL1* mutations are the commonest known mutations in advanced MDS, suggesting their important role in disease progression.

Conflict of interest

The authors declare no conflict of interest.

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AML with CBFB–MYH11 rearrangement demonstrate RAS pathway alterations in 92% of all cases including a high frequency of NF1 deletions

Acute myeloid leukemia (AML) with abnormal bone marrow eosinophils and with inv(16)(p13q22) or t(16;16)(p13;q22) leading to a CBFB–MYH11 fusion gene have been defined as a distinct entity by the World Health Organization classification and are associated with a favorable outcome. The CBFB–MYH11 fusion proteins are interfering in a dominant-negative manner with core binding factor (CBF), thereby impairing hematopoietic differentiation and predisposing cells to leukemic transformation. However, knock-in mouse models have demonstrated that CBFB–MYH11 alone is not sufficient to cause a leukemic phenotype and that additional aberrations were essential for the development of AML.[1] Secondary cytogenetic abnormalities occur in ∼40% of cases with +22, +8 (10–15% each), and del(7q) or +21 is most commonly observed. Recent molecular analyses have also provided important insights into the pathogenesis of myeloid disorders, and the commonly detected mutations of KIT and NRAS genes as well as deregulated CEBPA expression have been identified as likely candidates for cooperating events in CBF-positive AML.[2,3] Recently, exon 8 splice site mutations in the gene encoding the E3-ligase CBL have been reported in three cases with inv(16), suggesting an alternative mechanism of activating tyrosine kinases, as mutant CBL may act as a dominant-negative protein by inhibiting proper downregulation of activated tyrosine kinases, such as KIT and FLT3.[4]

This study was performed to further decipher accompanying genetic lesions at the molecular level in AML with CBFB–MYH11 rearrangements. Therefore, chromosome banding analyses, single-nucleotide polymorphism (SNP) array analyses and comprehensive screening for mutations in the genes KIT, NRAS, KRAS, FLT3, CBL, PTPN11 and JAK2 were performed in 37 newly diagnosed AML with CBFB–MYH11 rearrangement. Details on patient's characteristics are provided in the Supplementary Information. Patients gave their informed consent for this investigation. The study abides by the rules of the local Internal Review Board and the tenets of the revised Helsinki protocol. Chromosome banding analysis was available in all cases. In total, 30 cases showed an inv(16)(p13q22), whereas in 5 cases a t(16;16)(p13;q22) was observed. Two patients showed a normal karyotype with a cryptic CBFB–MYH11 rearrangement confirmed by reverse transcriptase PCR. Using standard chromosome banding analysis, additional cytogenetic aberrations were observed in 13 cases with de novo AML and in 1 case with t(therapy-related)-AML (37.8%). Karyotypes of patients with additional chromosome aberrations are depicted in the Supplementary Information (Supplementary Table S1). Recurring additional aberrations were +22 (n=6), +8 (n=5) or +8q (n=1), +13 (n=1) or +13q (n=1), −Y (n=2) and del(7q) (n=2). Gains and losses as well as large regions of copy-neutral loss of heterozygosity detected by SNP array analysis are depicted in detail in Table 1. Detailed information on chromosomal gains and losses identified by SNP arrays as compared with chromosome banding analysis are provided in the Supplementary Information. A 7q deletion was observed in a total of four cases. Two of these were detected by SNP array analyses only; they were missed by cytogenetics because of their small size. On the basis of SNP array data, the commonly deleted region was identified to range from 7q36.1 to 7q36.3