proportions ranging from 0% to 38% have also been reported, depending on the criteria for the excessive Lyonization definition, the type of XCIP, and even the tissue type. However, given that CIN patients and healthy controls are age-matched, the differences in clonality assessment obtained should be taken into account as they cannot be attributed to the influence of age-related skewing. Furthermore, no significant difference was found in age distribution between subjects (patients or controls) with clonal and those with non-clonal XCIP, suggesting that our results are unlikely to be biased by age.

The pathophysiological significance of the clonal populations identified in CIN patients is obscure. They might indicate a contraction of the stem cell pool resulting in support of hematopoiesis by one or a few stem cells, as previously described in aplastic anemia and hypoplastic myelodysplastic syndromes (MDS).[4] Alternatively, they might imply an early event in a transformation process and although malignant evolution has been rarely reported in CIN, a thorough long-term follow up of patients with clonal populations is needed.[12] The clonal XCIP in our patients should not be interpreted as indicative for MDS diagnosis because the majority of these patients had a long history of stable disease. Overall, we have provided evidence for possible clonal hematopoiesis in CIN. However, clonality data cannot be currently used as a diagnostic criterion for the disease and further studies with newer technologies, such as comparative genomic hybridization and single nucleotide polymorphism array-based karyotyping, are required to confirm clonal patterns of hematopoiesis in these patients.

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ETV6 deletion is a common additional abnormality in patients with myelodysplastic syndromes or acute myeloid leukemia and monosomy 7

Chromosome 7 abnormalities are common in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). Often, there is partial loss of 7q as the result of deletion (del(7q)) or loss of one entire copy of chromosome 7 (monosomy 7 or -7). Del(7q) and -7 may occur in isolation, in simple or in complex karyotypes.[1] It is assumed that the pathogenicity of -7/del(7q) is due to loss of tumor suppressor gene(s). However, the molecular mechanisms underpinning the clinical behavior of patients with 7/del(7q) MDS/AML are not well understood.

Deregulation of the *ETV6* gene through translocation, deletion or somatic mutation is also recurrent in myeloid malignancies. By metaphase cytogenetics (MC), approximately 1% of patients with AML and 0.6% of patients with MDS have deletion of part of 12p.[2,3] Mapping studies place *ETV6* within the smallest commonly deleted region.[4]

It has been reported that 5% of patients with -7/del(7q) have del(12p) by MC.[1] Using a single nucleotide polymorphism array (SNP-A), we identified a cryptic *ETV6* deletion in a patient with -7. The poor chromosome morphology typically encountered in patients with -7 means that it may be difficult to identify subtle 12p abnormalities in metaphases. We, therefore, reasoned that the frequency of *ETV6* deletion in patients with -7 may be higher than

is currently appreciated. In order to test this hypothesis, we performed fluorescence *in situ* hybridization (FISH) using an allele-specific *ETV6* probe in patients with -7.

We used the Victorian Cancer Cytogenetics Service database to identify samples from patients with -7 (either as the sole autosomal abnormality or in association with an abnormality of 12p) and a diagnosis of MDS, myelodysplastic/myeloproliferative neoplasm (MDS/MPN) or AML. Thirty-eight patients referred for testing between 1st January 2000 and 1st January 2011 had residual cytogenetic suspension suitable for FISH. Two patients had a 12p abnormality. In 36 cases, -7 was the only autosomal abnormality. Twenty-one patients had a diagnosis of MDS or MDS/MPN. The remainder had a diagnosis of AML.

We identified *ETV6* deletion in 6 of 38 (16%) cases. Patients with an *ETV6* deletion had a median age of 51.5 years (range 24-95) (Table 1). In 5 of 6 cases, the *ETV6* deletion was cytogenetically cryptic. The percentage of cells with *ETV6* loss ranged from 23% to 81% and was higher in AML than in MDS (average±SEM 76.2±2.7% *vs.* 35.3±9.5%, P=0.02) even though the majority of metaphases in all patients were derived from the -7 clone. *ETV6* deletions were heterozygous in all cases.

Some abnormal FISH signal patterns were consistent with complete loss of one *ETV6* allele; others suggested partial loss with a breakpoint positioned within the *ETV6* locus (Table 1). A simple signal pattern consistent with complete deletion of one copy of *ETV6* (loss of one 5' and 3' *ETV6* signal per cell) was the only abnormality detected in 4 patients (Figure 1A). In SVHM5 there was loss of one 3' signal with retention of the 5' signal in all abnormal cells consistent with a partial deletion where the distal breakpoint on 12p was positioned downstream of the area recognized by the 5' probe (Figure 1B). The remaining patient, SVHM4, had two different cell populations. A 5' signal of diminished intensity was present together with loss of one 3' signal per cell in 51.5% cells scored suggesting that the telomeric breakpoint of the deleted allele was located within the region recognized by the 5' probe. In another 20% of cells, one entire copy of both the 5' and 3' signals was lost, indicating breakpoints located outside *ETV6* (Figure 1C-E). Thus, there was heterogeneity with respect to the breakpoints relative to the position of the *ETV6* locus.

Two patients had genomic DNA collected allowing SNP-A karyotyping to be performed. In SVHM1, the SNP-A demonstrated a 1.3 Mb mono-allelic deletion encompassing *ETV6* (Figure 1F and Table 1). In SVHM3, the SNP-A delineated a 7.2 Mb heterozygous deletion spanning the entire *ETV6* locus (Table 1). Strikingly, in both cases, no additional cytogenetically cryptic somatic copy number changes or UPD were identified.

SVHM3 was the only case where paired diagnostic and follow-up samples were available. In this patient, *ETV6* deletion was not observed at diagnosis but was detected in a sample obtained five and a half months later (Table 1). On the balance of probability, given that the -7 clone is larger and that either acquisition of the *ETV6* deletion or expansion of an occult *ETV6* deleted clone occurred in the months between diagnosis and reassessment, it is likely that in this case -7 preceded development of the *ETV6* lesion.

Previous reports place the prevalence of *ETV6* deletion in MDS/AML somewhere between 0% and 64% with the frequency heavily dependent on the characteristics of the study population. Large representative studies in MDS and AML consistently find rates of less than 5%.[5-7] The highest rates occurred in cohorts enriched for patients with structural or numerical abnormalities of chromosome 12.[8,9] Interestingly, in other studies where the rate of *ETV6* deletion exceeded 5%, there was a strong association with complex karyotypes that fre-

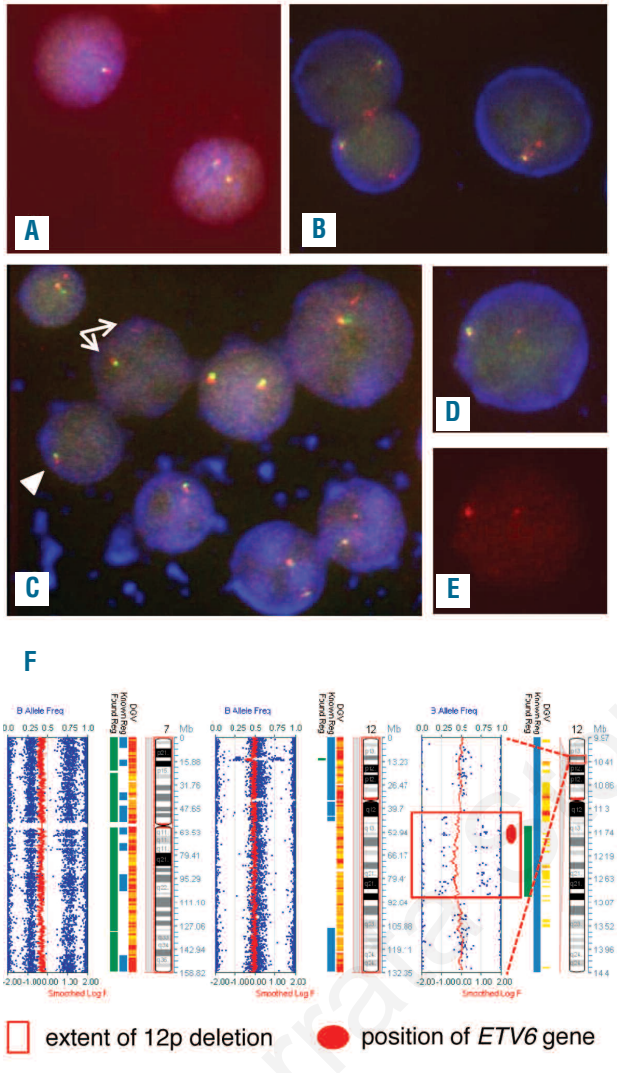

Figure 1. Representative FISH and SNP-A results for patients with *ETV6* deletion. (A) An interphase cell with one *ETV6* fusion signal (top left) consistent with complete loss of one *ETV6* allele and a cell with two *ETV6* fusion signals (bottom right) in SVHM3. The same signal patterns were observed in SVHM1, SVHM2 and SVHM6. (B) One *ETV6* fusion signal (5' and 3' *ETV6*) and one red 5' *ETV6* signal per cell in three interphase cells from SVHM5. (C) A mixed population of cells, some with one *ETV6* fusion signal and one red 5' *ETV6* signal of diminished intensity (white arrows) and one cell with a single *ETV6* fusion signal only (white arrowhead) in SVHM4. (D and E) The diminished intensity of the red signal on the deleted *ETV6* allele in SVHM4 was more readily apparent on the single color (Spectrum Orange) image (E) than on the triple color (DAPI/FITC/Spectrum Orange) image of the same cell (D). Fluorescent signals were visualized under an Axioplan 2 (Zeiss) microscope and captured and analyzed using ISIS image analysis software (Metasystems, Altlussheim, Germany). (F) SNP-A abnormalities in SVHM1. Smoothed log R values (red) and B allele frequencies (blue) for chromosome 7 (whole chromosome view, left panel), chromosome 12 (whole chromosome view, middle panel) and the deleted region of 12p (right panel) with the extent of deletion shown by the red box and the position of the *ETV6* gene indicated by the red circle. DNA (200 ng) was hybridized to CytoSNP-12 BeadChip arrays (Illumina, San Diego, CA, USA) and analysis was performed using Karyostudio v1.2 software (Illumina).

Table 1. Characteristics and karyotypes of patients with an *ETV6* deletion.

ID	Age (years)	Diagnosis	Karyotype	*ETV6* deletion (% cells)	Complete/ partial *ETV6* deletion
SVHM1	95	MDS/RCMD	45,XX,-7[19].nuc ish(ETV6x1)[106/197].arr 7p22.3q36.2(141,322-158,812,247)x1~2, 12p13.2p13.1(11,646,252-12,981,168)x1~2	54.0	Complete
SVHM2	36	MDS/ unknown	45,XY,-7[9]/ 45,idem,del(12)(p11)[11]. nuc ish(ETV6x1)[46/200]	23.0	Complete
SVHM3 (Diagnosis)	62	MDS/ RCMD	45,XX,-7[11]/ 46,XX[11].nuc ish(ETV6x2)[200]	None	None
SVHM3 (Follow up)	63	MDS/ RCMD	45,XX,-7[21]/46,XX[4].nuc ish(D7Z1,FRA7G)x1 [86/100],(ETV6x1)[58/200]. arr 7p22.3q36.3(141,322-158,812,247)x1~2, 12p13.31p12.3(10,596,649-17,742,422)x1~2	29.0	Complete
SVHM4	85	AML (previous CMML)	45,XY,-7[40].nuc ish(ETV6x1)[40/200]/ (5'ETV6x1,5'ETV6 dim x1,3'ETV6x1) (5'ETV6 con 3'ETV6x1)[103/200]	71.5	Partial: part of 5' *ETV6* retained (51.5%), complete (20%)
SVHM5	40	AML	45,XX,-7[21].nuc ish(5'ETV6x2,3'ETV6x1) (5'ETV6 con 3'ETV6x1)[152/200]	76.0	Partial: 5' *ETV6* retained
SVHM6	24	AML	45,XY,-7[20]/ 46,XY[1].nuc ish(ETV6x1)[162/200]	81.0	Complete

MDS: myelodysplatics syndromes; RCMD: refractory cytopenia with multilineage dysplasia; AML: acute myeloid leukemia; CMML: chronic myelomonocytic leukemia.

quently exhibited -7/del(7q).[10-12]

Outside of two cohorts with chromosome 12 abnormalities[8,9] and one case series with complex karyotypes,[12] our study has the highest reported frequency of *ETV6* deletion. At 16%, the prevalence of *ETV6* loss in -7 patients is in excess of 3 times the expected rate in unselected MDS/AML cohorts. Although the association between -7/del(7q) also holds up in complex karyotypes in published series[10-12] and in our own experience (R. MacKinnon *et al.*, unpublished data, 2012) the presence of gross chromosomal instability in that setting can make it difficult to distinguish driver from passenger lesions. Co-occurrence of just two copy number changes, at rates higher than might be expected by chance in this study is more compelling evidence in support of functional cooperation between -7 and *ETV6* deletion.

As we enter the era of molecularly targeted therapy, it is important to understand the consequences of genetic events that are critical for maintenance and progression of malignant phenotypes. Our results suggest that -7 and *ETV6* loss drive complementary steps during leukemogenesis.

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**MW and KCR contributed equally to this manuscript.*

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Mutations in *DNMT3A* and loss of RKIP are independent events in acute monocytic leukemia

Methylation of DNA in CpG-rich islands is a key event in the regulation of tissue- and context-specific gene expression. Thereby, DNA methyltransferase 3A (*DNMT3A*) plays a pivotal role by converting cytosine to 5-methylcytosine.[1] Recently, *DNMT3A* mutations have been described at high frequency in acute myeloid leukemia (AML), particularly with a monocytic phenotype.[2-4] In *in vitro* assays, mutated *DNMT3A* induced aberrant DNA methylation and promoted cellular proliferation. RAF kinase inhibitor protein (RKIP) negatively regulates the RAS-mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway.[5] Several solid neoplasms show decreased or absent expression of RKIP and its role as a metastasis suppressor has been firmly established in animal models and studies of human tumors.[5] We recently demonstrated that RKIP acts as a tumor suppressor in hematopoietic cells and that loss of RKIP expression occurs frequently in AML.[6,7] Similar to mutated *DNMT3A*, RKIP loss is linked to AMLs with a monocytic phenotype. We, therefore, asked whether loss of RKIP and mutations in *DNMT3A* correlate in this particular subtype.

For PCR and direct sequencing of *DNMT3A*, its coding sequences and splice sites comprising 22 exons were analyzed as previously described[5] in leukemic specimens of 36 patients diagnosed with acute monocytic leukemia; patients were classified in subgroups M4 and M5 according to the French-American-British (FAB) classification. Cytogenetic information was available in 32 patients with 4 of 32 (12.5%) karyotypes conferring good, 21 of 32 (65.5%) intermediate, and 7 of 32 (22%) adverse risk. Median age at diagnosis was 55.5 years (range 18-80 years), median white blood cell (WBC) count 55.5x10^9/L (range 5-445x10^9/L). This cohort has also been characterized for RKIP expression, showing its loss in 17 of 36 (47%) patients as determined at protein and mRNA level using Western blot and quantitative real-time polymerase chain reaction (PCR), respectively.[7] Screening for mutations in *NRAS* and *KRAS* (codons 12, 13 and 61) as well as in *NPM1* (exon 12) has been performed as previously described.[7,8] Detection of *FLT3* internal tandem duplications (ITD) and tyrosine kinase domain (TKD) mutations was performed using the *FLT3* Mutation Assay (*InVivo*Scribe Technologies, San Diego, CA, USA) according to the manufacturer's protocol. Informed consent was obtained from all individuals and the study was approved by the institutional review board of the Medical University of Graz, Austria. Statistical correlations were calculated by Fisher's exact (for correlation of *DNMT3A* with *RKIP*, *FLT3* and *NPM1*) and by Mann-Whitney-Wilcoxon test (for correlation of *DNMT3A* with cytogenetics, WBC count and age at diagnosis), using R 2.15.1 (*http://www.r-project.org*).

We detected mutations in *DNMT3A* in 16 of 36 (44%) patients with monocytic AML. This high frequency is consistent with previous reports on *DNMT3A* mutations in this AML subtype.[2,4] Fifteen of these mutations constituted the recently described hot spot mutations R882H and R882C, respectively.[2,4] In one sample, we detected an R676W substitution, a *DNMT3A* mutation that has not yet been described. Its somatic origin could be proven by analysis of constitutional material (Figure 1). This substitution was predicted to be "disease causing" by MutationTaster[9] and "damaging" by Sorting Intolerant From Tolerant (SIFT).[10] WBC counts at diagnosis were significantly higher in patients with mutant as compared to wild-type *DNMT3A* (77x10^9/L *vs*. 51x10^9/L; *P*=0.040), whereas no significant difference could be observed for cytogenetic risk groups and patients age at diagnosis (*data not shown*). In the cohort investigated, *DNMT3A* mutations were associated with alterations of *NPM1* as 11 of 14 (79%) *DNMT3A* mutated patients also harbored a *NPM1* mutation compared to 6 of 20 (30%) *DNMT3A*

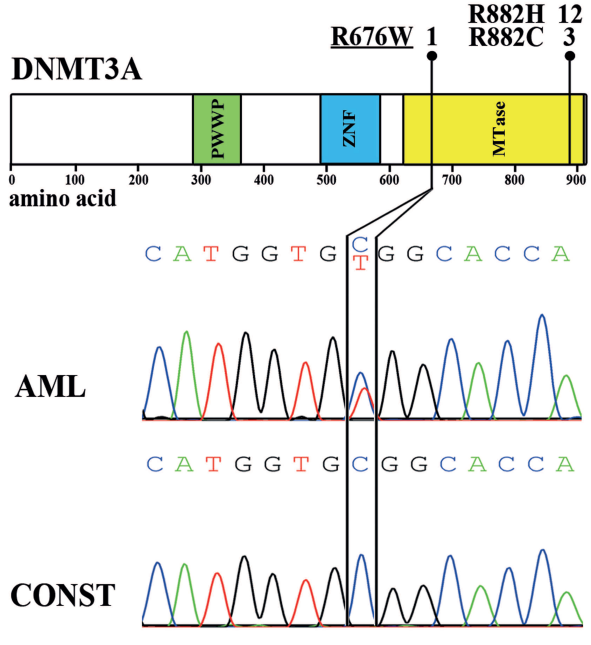

Figure 1. R676W is a novel somatic DNMT3A mutation. Number of distinct mutations detected in this study and their locations within the DNMT3A protein. The novel R676W substitution is caused by the heterozygous C2026T substitution as highlighted with black bars in the electropherogram of leukemic DNA. Absence of this mutation in constitutional material proves its somatic origin. PWWP: proline-tryptophan-tryptophan-proline domain; ZNF: zinc finger domain; MTase: methyltransferase domain; CONST: constitutional material.