Identification of Two Critically Deleted Regions within Chromosome Segment 7q35-q36 in EVI1 Deregulated Myeloid Leukemia Cell Lines

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Abstract

Chromosomal rearrangements involving the EVI1 proto-oncogene are a recurrent finding in myeloid leukemias and are indicative of a poor prognosis. Rearrangements of the EVI1 locus are often associated with monosomy 7 or cytogenetic detectable deletions of part of 7q. As EVI1 overexpression alone is not sufficient to induce leukemia, loss of a 7q tumour suppressor gene might be a required cooperating event. To test this hypothesis, we performed high-resolution array comparative genomic hybridization analysis of twelve EVI1 overexpressing patients and three EVI1 deregulated cell lines to search for 7q submicroscopic deletions. This analysis lead to the delineation of two critical regions, one of 0.39 Mb on 7q35 containing the CNTNAP2 gene and one of 1.33 Mb on chromosome bands 7q35–q36 comprising nine genes in EVI1 deregulated cell lines. These findings open the way to further studies aimed at identifying the culprit EVI1 implicated tumour suppressor genes on 7q.

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Introduction

Chromosomal rearrangements involving chromosome band 3q26, such as translocations with various partner chromosomes or inversions of chromosome 3 are a recurrent finding in myeloid leukemias [1]. These aberrations contribute to the ectopic expression of the EVI1 proto-oncogene. EVI1 transcriptional activation has been reported in up to 10% of myeloid leukemia patients, even in the absence of 3q26 rearrangements, and is an independent indicator of adverse prognosis [2].

Retroviral integration experiments have shown that EVI1 overexpression alone is not sufficient to cause leukemia, indicating that cooperative effects are necessary for malignant transformation [3,4]. Interestingly, over fifty percent of the 3q26 rearranged leukemias also display chromosomal abnormalities involving chromosome 7, such as monosomy 7 or deletion of part of 7q [5]. This association alludes to the existence of a 7q tumour suppressor gene, which when deleted acts in concert with EVI1 overexpression to induce malignant transformation. We performed high-resolution array comparative genomic hybridization (CGH) analysis to search for 7q submicroscopic deletions in EVI1 deregulated leukemia patients in order to identify candidate 7q tumour suppressor genes.

Materials and Methods

Patients and Cell Lines

Diagnostic bone marrow samples of twelve myeloid leukemia samples and remission bone marrow samples of two patients (case 7 and case 8) were included in the study. Fluorescence in situ hybridization (FISH) for detection of EVI1 rearrangement and reverse transcription quantitative PCR (RT-qPCR) for detection of EVI1 ectopic expression was performed as previously described [6]. The study was approved by the ethics committee of the Ghent University Hospital (2003/273).

Three EVI1 rearranged cell lines, Kasumi-3, MUTZ-3 and UCSD-AML1 were also included in the study [7,8]. For the cell lines culture conditions were as follows, for Kasumi-3 RPMI-1640 medium (Invitrogen, Belgium) was supplemented with 15% foetal calf serum, 1% penicillin/streptomycin, 1% kanamycin, 1% glutamine, 2% HEPES (1 M), 1% sodiumpyruvate (100 nM) and 0.1% beta-mercapto ethanol (50 nM). For MUTZ-3, the used medium contained an extra 10% of the supernatant of the 5637 urinary bladder carcinoma cell line [9] and 10 ng/ml of GM-CSF (Promocell, UK). For the UCSD-AML1 cell line, the used medium contained an additional 10 ng/ml of GM-CSF (Promocell, UK).
Table 1. Patient and cell line characteristics: diagnosis, karyotype and EVI1 FISH and RT-qPCR results.

| Name       | Diagnosis*       | Karyotype†          | FISH EVI1‡ | RT-qPCR EVI1‡ |
|------------|------------------|---------------------|------------|---------------|
| Kasumi-3   | AML              | 46,XY,t(2;3)(p13;q33),t(3;7)(q26;q22).del(5)(q15),+,8.del(9)(q32),add(12)(p11).add(16)(q13),+mar[20] | +          |              |
| MUTZ-3     | AML              | 46,XY,t(1;3)(q43;p13.11).t(2;7)(q36;p36).inv(3)(q21q26).inv(7)(p15q36).t(12;22)(p13;q12)[20] | +          | +            |
| UCSD-AML1  | AML              | 45,XX,t(22;21)(p13;q12),t(3;3)(q21q26),−7[20] | +          | +            |
| case 1 MDS |                  | 46,XX,t(3;8)(q26;q23)[13]/46,XX[1] | +          |              |
| case 2 AML |                  | 47,XY,+8.inv(3)(q21q26).t(13;22)(q10;p10)[20] | +          | +            |
| case 3 AML |                  | 46,XY,t(3;21)(q26;q22).add(17)(p11.2)[12]/46,XY[5] | +          | +            |
| case 4 MDS |                  | 45,XX,add(5)[q13].t(3;21)[q26;q22].add(5)[q13],−13,−18,+22[10]/46,XY[6] | +          | +            |
| case 5 AML |                  | 46,XY.inv(3)[q21q26][8]/46,XY[2] | +          | +            |
| case 6 AML |                  | 47,XX,t(9;11)(p22;q23).del(11)[p15],[+21][8]/46,XX[12] | −          | +            |
| case 7 AML |                  | 46,XX,t(9;11)[p22;q23][8]/46,XX[12] | −          | −            |
| case 8 AML |                  | 45,XX,+X.t(9;11)[p22;q23][12]/46,XX[6]/45,XX,−X[3] | −          | +            |
| case 9 AML |                  | 45,XY.inv(3)[q21q26],−7[17] | +          | +            |
| case 10 AML|                  | 46,XY,t(3;3)[q21q26][15] | +          | +            |
| case 11 AML|                  | 45,XX,add(3)[q26],−7[15] | +          | +            |
| case 12 AML|                  | 45,XY,t(3;21)[q26;q22],−7[20] | −          | +            |

Bold formatting indicates the 3q26 rearrangement.
†AML = acute myeloid leukemia and MDS = myelodysplastic syndrome.
‡The chromosomal aberration implicating the EVI1 locus is indicated with bold formatting.
§Positive for EVI1 FISH/qRT-PCR = + and negative for EVI1 FISH/qRT-PCR = −.

Patient and cell line characteristics and EVI1 FISH and RT-qPCR results are described in Table 1.

Array CGH

DNA of patient samples and cell lines was isolated using the QIAamp DNA mini kit (Qiagen, Belgium) or the Puregene Cell kit (Gentra Systems, Belgium) according to the manufacturer’s descriptions. Array CGH analysis for submicroscopic 7q deletions was performed on a 44K custom array (Agilent Technologies, Belgium) covering the entire chromosome 7 with a probe spacing of 1 oligonucleotide every 3 kb, according to the manufacturer’s descriptions. In brief, DNA (400 ng) was labelled using the BioPrime Array CGH genomic labelling system (Invitrogen, Belgium) using Cy5 (control DNA; Promega, Belgium) and Cy3 (patient sample or cell line) labelled dCTPs (GE Healthcare, Belgium). Following labelling, hybridization, and washing of the slides, arrays were scanned using an Agilent DNA Microarray Scanner, quantified with Feature Extraction software 10.1 and data were further analyzed with arrayCGHbase [10] using a circular binary segmentation (CBS) algorithm taken into consideration log2 ratios of neighbouring probes [11]. Deletions were called heterozygous when CBS ratios were ≤ −0.5 and a deletion was considered putatively homozygous when the CBS ratios were ≤ −1.2. Common copy number variations (CNVs) present in the Database of Genomic Variants (www.projects.tcag.ca/variation) were excluded from analysis.

Fluorescence In Situ Hybridization

To confirm the overlapping deletions found in the cell lines Kasumi-3, UCSD-AML1 and MUTZ-3 at 7q55-q36, FISH analysis was performed as previously described [6]. FISH probes were selected from the UCSC genome browser database (http://genome.ucsc.edu) (Table 2).

CNTNAP2 Expression Analysis

Total RNA was extracted from total bone marrow samples of thirty nine EVI1 overexpressing patients (nine with monosomy 7), five normal bone marrow samples and two CD34+ cell fractions, using the miRNeasy kit (Qiagen, Belgium). cDNA was prepared from 2 µg of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad, Belgium) according to the manufacturer’s descriptions and RT-qPCR for the CNTNAP2 (forward: 5’-TAGGACATGGA-ACCCCAATG-3’ and reverse: 5’-ATCGATTTGGCTCATCTTTGG-3’) transcript was performed as previously described [12,13]. The reference genes RPL13A (forward: 5’-CCTGGAGAGAGAGAGAGA-3’ and reverse: 5’-TTGAGGACC- TCGTGTATTGGTCCA-3’) and YWHAZ (forward: 5’-AC- TTGGTACATTGTGGCTTCAA-3’ and reverse: 5’-CCGGC- CAGGACAAACCAGTAT-3’) were used for normalisation of the RT-qPCR data.
Results and Discussion

Based on karyotyping and FISH analysis, two critical regions, one on chromosome band 7q22 [14] and one on chromosome bands 7q32–q35 [15], have been identified as the targets for 7q deletions in myeloid leukemia. In this study, array CGH was applied to search for deletions on chromosome 7 at ultra-high resolution. Using this approach, we identified several submicroscopic 7p and 7q deletions in EVII overexpressing patients and cell lines (Table 3 and Figure 1) including two critically deleted regions on 7q35–q36 in lines (Table 3 and Figure 1) including two critically deleted regions.

Between brackets is the total number of genes residing in the deleted area.

However, upon RT-qPCR analysis of the CNTNAP2 gene no expression could be detected in normal bone marrow samples, CD34+ cell fractions or EVII overexpressing bone marrow samples (data not shown), indicating that this gene is not a straightforward candidate 7q tumour suppressor gene cooperating with EVII overexpression.

Subsequent screening of twelve EVII overexpressing patient samples with (3 cases) or without (9 cases) monosomy 7, revealed no submicroscopic alterations within the above described SROs. However, focal deletions in other genomic regions on chromosome arms 7p and 7q were detected.

Of interest is that both the Kasumi-3 and the MUTZ-3 cell line display chromosomal rearrangements involving chromosome 7. It is well known that small deletions can occur at chromosomal breakpoints [18]. Therefore, the observed 4.83 Mb deletion at 7q36 in MUTZ-3 might have originated as a consequence of the t(2;7)(q36;q36) and/or the inv(7)(p15q36) present in this cell line.

Of the nine genes located within the 1.33 Mb SRO CUL1 and EZH2 are the most promising candidates due to known function in association with cancer. The CUL1 gene encodes a protein that, when associated with other proteins such as Skp1, forms the Skp2 E3 ubiquitin ligase E3 complex involved in degradation of different proteins [19]. In AML, the Skp2 complex has been described to target the MEF transcription factor which induces G1/S transition. Impairment of the Skp2 complex leads to a decrease in MEF transcription factor which induces G1/S transition. Impairment of the Skp2 complex leads to a decrease in MEF degradation thereby promoting cell proliferation [20]. Deletion and mutation of other E3 ubiquitin ligase complex members such as the F-box containing FBXW7 gene has already

Table 3. Array CGH results of chromosome 7 deletions.

| Name          | Chromosomal position* | Start - end (Mb) | Size (Mb) | Genes† |
|---------------|------------------------|------------------|-----------|--------|
| Kasumi-3     | 7p22                   | 2369956–6743766  | 4.37      | SDK1 (40) |
|              | 7p22–p15               | 7010834–26480795 | 19.47     | 63     |
| 7q31.2       | 116412304–116584949    | 0.17             | ST7       |
| 7q34–q36     | 142182589–148693649    | 6.51             | KEL, CNTNAP2, CUL1, EZH2 (48) |
| MUTZ-3       | 7q35–q36               | 147377325–152205764 | 4.83 | KEL, CNTNAP2, CUL1, EZH2 (61) |
| UCSD-AML1    | 7q11.22                | 69266162–69318942 | 0.053 | AUTS2† |
|              | 7q21.11                | 78183200–78189877 | 0.007 | MAGI2† |
|              | 7q35                   | 146482743–146873259 | 0.391 | CNTNAP2† |
| case 2       | 7q34                   | 142351441–142361904 | 0.01  | KEL    |
| case 3       | 7p22                   | 4036389–4042650   | 0.01    | SDK1   |
|              | 7p12                   | 43592619–43597672 | 0.01    | STK17A |
| case 9       | 7q32.2                 | 120976051–120978116 | 0.02  | NRF1†  |

Bold formatting indicates deletions on the 7q arm.

*Based upon the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway, NCBI Build 36.1).

†Between brackets is the total number of genes residing in the deleted area.

†Homozygous deletion.

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Figure 1. Overview of deletions in cell lines and patients. Chromosome view of chromosome 7 deletions in the cell lines Kasumi-3, MUTZ-3 and UCSD-AML1 and in cases 2, 3 and 9. Deletions are indicated using grey bars.

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been described in T-cell acute lymphoblastic leukemia (T-ALL) where it is associated with poor prognosis [21]. Moreover, in AML loss-of-heterozygosity (LOH) of the \( \text{EZH2} \) (polycomb group) gene (enlarger of zest) was detected in 5 out of 21 patients [22]. However, further analyses such as mutation screening and functional RNA interference screens [23] are needed to identify the genes contributing to \( \text{EVI1} \) leukemogenesis.

In addition to the regions described above, other 7q deletions in the \( \text{EVI1} \) overexpressing patients as well as in the UCSD-AML1 cell line were detected. In UCSD-AML1 two additional small 7q deletions were observed, a 53 kb (7q11.22) and 7 kb (7q21.11) deletion and containing the \( \text{AUTS2} \) and \( \text{MAGI2} \) genes respectively. In case 2, a small deletion of 10 kb on 7q34 was observed encompassing the \( \text{KEL} \) blood group gene. In Kasumi-3, this gene was located in a larger deleted region of 4.37 Mb on 7p22. As involvement of the \( \text{SDK1} \) gene in apoptosis has already been described [25], deletion of this gene could lead to a decreased rate of cell death. For patient 3 deletion of the \( \text{STK17A} \) gene at 7p12 was detected. Deletion of this pro-apoptotic gene had already been described in laryngeal squamous cell carcinoma [26]. It should be noted that it is possible that the above described small 7q and 7p deletions in patient samples are not leukemogenic as no constitutional material of these patients was available. In the remaining patient samples no chromosome 7 deletions could be detected.

In conclusion, using high-resolution array CGH, we were able to delineate two critical regions for 7q deletions in myeloid leukemias that were not detectable using standard karyotyping. Besides a 0.39 Mb SRO on 7q35 containing the \( \text{CNTNAP2} \) gene, we identified a 1.33 Mb region on chromosome bands 7q35–q36 containing nine putative tumour suppressor genes in \( \text{EVI1} \) deregulated cell lines. Further analysis of these candidates is warranted to investigate their role in \( \text{EVI1} \) mediated malignant

Figure 2. FISH analysis of the 7q35–q36 deletions in cell lines. A), D) and G) FISH analysis on cytogenetically normal controls with probes located within the 7q35–q36 deleted regions. B), E) and H) FISH analysis with probes located within the 7q35–q36 deleted regions on the cell lines Kasumi-3 (B), UCSD-AML1 (E) and MUTZ-3 (H). C), F) and I) FISH analysis with probes just outside of the 7q35–q36 deleted regions on the cell lines Kasumi-3 (C), UCSD-AML1 (F) and MUTZ-3 (I).
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Figure 3. Array CGH profile of chromosome 7 (141 Mb–153 Mb) for Kasumi-3, MUTZ-3 and UCSD-AML1. A) Array CGH profile of Kasumi-3, B) MUTZ-3 and C) UCSD-AML1 indicating the 0.39 Mb SRO on 7q35 and the 1.33 Mb SRO on chromosome bands 7q35–q36. Log2-ratios of the clones are depicted by vertical dots corresponding to the respective genomic position (NCBI build 36). Deletions are shown in red. The SROs are indicated with a purple box. The bottom of the figure shows the genomic position with an indication of known CNVs as present in the Database of Genomic Variants (http://projects.tcag.ca/variation/). Regions of segmental duplication are displayed using black and grey boxes indicating identical genomic regions. A screenshot of the UCSC Genome Browser (NCBI build 36, http://genome.ucsc.edu) shows an overview of the known RefSeq genes in the 1.33 Mb critical region.

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7q Deletions in EVI1 Leukemia
Table 4. Genes located in the 1.33 Mb SRO on 7q35–q36.

| Gene name | Annotation |
|-----------|------------|
| CNTNAP2  | contactin associated protein-like 2 isofrom b |
| LOC392145| hypothetical protein LOC392145 |
| C7orf33  | hypothetical protein LOC202865 |
| CUL1     | cullin 1 |
| EZH2     | enhancer of zeste homolog 2 |
| PDK4     | protein disulfide isomerase associated 4 |
| ZNF786   | zinc finger protein 786 |
| ZNF425   | zinc finger protein 425 |
| ZNF398   | zinc finger 398 isofrom a |

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Author Contributions

Conceived and designed the experiments: ADW BP PVV ADP NVR BM FS. Performed the experiments: ADW SV. Analyzed the data: ADW PVV. Contributed reagents/materials/analysis tools: MP RDB YB LN. Wrote the paper: ADW BP PVV ADP NVR BM FS.