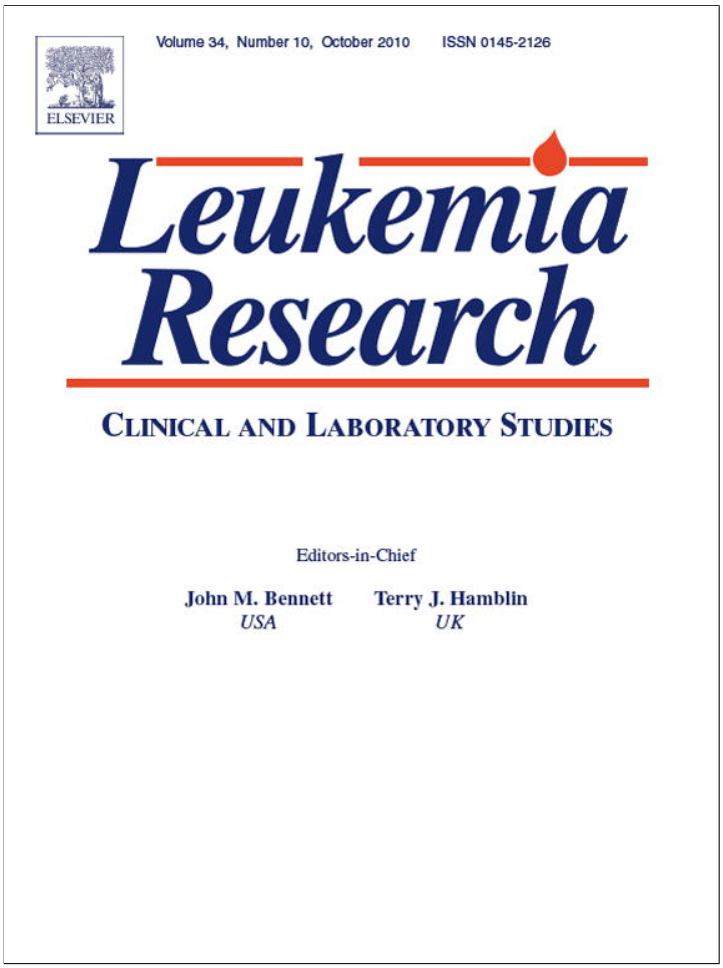

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Differential cytogenomics and miRNA signature of the Acute Myeloid Leukaemia Kasumi-1 cell line CD34$^+$38$^-$ compartment

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ABSTRACT

The t(8;21) Acute Myeloid Leukaemia (AML) Kasumi-1 cell line with N822K *KIT* mutation, is a model system for leukemogenesis. As AML initiating cells reside in the CD34$^+$CD38$^-$ fraction, we addressed the refined cytogenomic characterization and miRNA expression of Kasumi-1 cell line and its FACS-sorted subpopulations focussing on this compartment. By conventional cytogenetics, Spectral-Karyotyping and array-CGH the cytogenomic profile of Kasumi-1 cells evidenced only subtle regions differentially represented in CD34$^+$CD38$^-$ cells.

Expression profiling by a miRNA platform showed a set of miRNA differentially expressed in paired subpopulations and the signature of miR-584 and miR-182 upregulation in the CD34$^+$CD38$^-$ fraction.

1. Introduction

Accumulating evidences have shown that tumors, although monoclonal in origin, are composed of a heterogeneous population of cells that differ in their state of abnormal differentiation. A current explanation for the intra-tumor diversity is provided by the cancer-initiating cells (CICs) model that predicts a cellular hierarchy in which cells with self-renewal and differentiation ability coexist with more mature cells [1].

CICs have been differentially isolated from human cancers including leukaemia and different types of solid tumors [2]. In the case of AML it has been shown that the cell able to originate AML in the NOD-SCID mouse model (named the SL-IC, SCID Leukaemia Initiating Cell) has the CD34$^+$CD38$^-$ immunophenotype [3]. For some solid tumors like brain and breast tumors, CICs have been successfully isolated from immortalized cells [4,5], making cell lines a faithful model of the primary tumors and hence a suitable investigation system.

The AML Kasumi-1 cell line has the t(8;21)(q22;q22) translocation, a cytogenetic hallmark of M2 AML [6] originating the AML1/ETO fusion protein which expression has been shown to exert a dominant-negative activity on myeloid differentiation [7]. Moreover the Kasumi-1 cell line is characterized by increased dosage of a mutated allele (N822K) of the *KIT* gene leading to a constitutively activated form of the stem cell factor receptor [8]. The presence of these two mutations renders the cell line a paradigm of the "two-hits" model for AML pathogenesis [9]. Based on the immunophenotype of both the original blasts of the patient and the established cell line, Kasumi-1 cells were proposed to derive from an early myeloid stem cell [10]. We thus reasoned that early passage Kasumi-1 cell line might contain a subset of leukaemia initiating cells within the CD34$^+$CD38$^-$ fraction, making worthwhile a targeted characterization of this compartment. Here we report on the detailed immunophenotypic and cytogenomic characterization of the total Kasumi-1 cell line and its FACS-sorted subpopulations, mainly focusing on the CD34$^+$CD38$^-$ fraction. Moreover we report on the miRNA profile of Kasumi-1 cell line and its fractions searching for differential expression of a few miRNAs in the CD34$^+$CD38$^-$ subpopulation.

2. Materials and methods

2.1. Cell culture

The human AML Kasumi-1 cell line (AML M2, AML1-ETO) was obtained from DMSZ (Braunschweig, Germany) and maintained in RPMI 1640 (PBI International,

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Milan, Italy) with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA).

2.2. Immunophenotypic characterization

Kasumi-1 cells, after washing and blocking, were stained with the following antibodies: Cy7-phycoerythrine (PE) conjugated anti-CD34, PE conjugated anti-CD38, allophycocyanin (APC) conjugated anti-CD117, PE conjugated anti-CD33, fluorescein-5-isothiocyanate (FITC) anti-CD3, CD4, CD8, CD14, CD16, CD19, CD20 and FITC conjugated anti-CD13, CD235a, CD41 (Becton Dickinson, BD Biosciences). The analysis was performed using a FacsCalibur instrument (Becton Dickinson, BD Biosciences).

2.3. Cell sorting

Kasumi-1 cells were stained with anti-CD34 and anti-CD38 antibodies as described above. Cell sorting was performed using a Facs Vantage SE Laser 488 nm Interprise Coherent flow cytometer (Becton Dickinson, BD Biosciences), and gates were set up to exclude not viable cells and debris. The purity of sorted fractions was assessed to ensure sort quality.

2.4. SKY analysis

Chromosome spreads of Kasumi-1 cells were prepared using standard air-drying methods. The chromosomal DNA, after digestion with RNase and pepsin according to the procedure recommended by Applied Spectral Imaging, Inc. (ASI: Vista, CA 92081) and denaturation, was hybridized with a cocktail of human SKY paint probes, as previously described [11]. The images of 56 mitoses chosen at random were captured with a Spectral cube and Interferometer module installed on a Nikon microscope. Spectral-Karyotyping was carried out using SKY View software (Version 1.62).

2.5. Nucleic acid extraction

DNA and RNA were isolated from FACS-sorted $CD34^-$, $CD34^+CD38^-$, $CD34^+38^+$ Kasumi-1 subpopulations and Kasumi-1 total cell line using the NucleoSpin Tissue kit (Macherey-Nagel, Gmbh & Co. KG) and Trizol reagents (Invitrogen Life Technologies, Cergy, France) respectively, according to the manufacturer's instructions. DNA and RNA quality and concentration were evaluated using spectrophotometer NANODROP 1000 (Thermo Fischer Scientific Inc.). DNA and RNA were conserved at −20 °C and −80 °C respectively until the use.

2.6. Array-CGH

An oligonucleotide array, containing 244,000 probes designed for human CGH, was utilized (Agilent Technologies, Palo Alto, CA). It is composed of 60-mer oligonucleotides at an average spatial resolution of 6.4 kb.

Labelling and hybridization of DNA: respectively 1 μg of reference DNA (pooled normal female control) and test sample genomic DNA (either Kasumi-1 total cell line and sorted $CD34^-$ and $CD34^+CD38^-$ cells) were individually fluorescently labelled using the Agilent Genomic DNA Labelling kit, Direct Method, as described by the manufacturer (Agilent Technologies, Palo Alto, CA). The labelled DNA was hybridized to the Agilent human 244K CGH array (Agilent Technologies, Palo Alto, CA) according to the manufacturer's instructions. Chip was scanned with an Agilent microarray scanner to generate high-resolution (5 μm) images.

Image and data analysis: Image analysis was performed using the Feature Extraction version 9.5.1.1 (Agilent Technologies, Palo Alto, CA) and the Agilent's CGH-v4_95_Feb07 protocol. The results of Feature Extraction were imported into CGH Analytics version 3.4.27 (Agilent Technologies, Palo Alto, CA). Aberration calls were made using Agilent's ADM-1 algorithm with a threshold of 10, comparing the Kasumi-1 DNA sample versus the control DNA sample.

2.7. Analysis of AML1/ETO transcripts

RNA from FACS-sorted $CD34^-$, $CD34^+CD38^-$, $CD34^+38^+$ Kasumi-1 subpopulations and Kasumi-1 total cell line was subjected to reverse transcription reaction using the Thermoscript RT-PCR kit (Invitrogen Life Technologies, Cergy, France). cDNA was amplified by AML1/ETO fusion transcript specific primers (forward: 5′GGCTGGCAATGATGAAAACT3′; reverse: 5′CGCCATTCAAGGCTGTAGGAG3′), gift of Dr. Myriam Alcalay, Ifom-Ieo campus, Milan, Italy.

2.8. Analysis of KIT mutation

DNA from Kasumi-1 total cell line and FACS-sorted $CD34^-$, $CD34^+CD38^-$, $CD34^+38^+$ subpopulations was analysed for the mutation in the exon17 of *KIT*. The analysis was performed as previously described [8].

2.9. miRNA microarray

The analysis was performed on Kasumi-1 and its subpopulations, sorted two times after the same propagation time in culture. Labelled RNA was hybridized on human miRNA microarray (#G4470B, Agilent Technologies, Palo Alto, CA) according to the manufacturer's procedure. This chip, consisting of 60-mer DNA probes, contains 15,000 features which represent 723 microRNAs, sourced from the Sanger miRBase database (Release 10.1). Images at 5 μm resolution were generated by Agilent scanner and the raw-data were obtained by Feature Extraction 10.5 software (Agilent Technologies, Palo Alto, CA).

2.10. Statistical and bioinformatics analysis

Microarray results were analysed using the GeneSpring GX 10 software (Agilent Technologies, Palo Alto, CA). Data transformation was applied to set all negative raw values at 1.0, followed by a quantile normalization. Sorted populations were normalized on the matched Kasumi-1 total population. A filter on low gene expression was used to keep only the probes expressed in at least one sample (flagged as Marginal or Present). Samples were grouped in accordance to their differentiation status and then compared. Differentially expressed genes were selected having a 1.5-fold expression difference between their geometrical mean and a statistically significant p-value (<0.05) using ANOVA (analysis of variance) statistic. Differentially expressed genes were employed for Cluster Analysis of samples using the Pearson correlation as a measure of similarity. Different bioinformatics tools were used to identify putative target genes predicted by at least two algorithms among TargetScanS 4.2 (http://www.targetscan.org/), Miranda (http://www.microrna.org/) and PicTar (http://www.pictar.org/).

2.11. Real Time PCR analysis of mature miR-584 and miR-182

In order to validate the data obtained by miRNA microarray, Real Time PCR analysis of miR-584 and miR-182 was performed on Kasumi-1 and its subpopulations ($CD34^-$, $CD34^+CD38^-$ and $CD34^+CD38^+$). Both miR-584 and miR-182 expression was evaluated by the $2^{-\Delta\Delta Ct}$ method [12] relative to RNU48 as endogenous normalization control. Both assays were provided by Applied Biosystems (TaqMan MicroRNA Expression Assay, ID# 001624 miR584, ID# 002334 miR182, 001006 RNU48). The qRT-PCR was performed with the TaqMan MicroRNA Assays Human Kit (Applied Biosystems, Foster City, CA), using Step-ONE Real Time PCR System (Applied Biosystems, Foster City, CA). Each sample was reverse transcribed in duplicate and cDNA was run in triplicate for both miRNAs and RNU48, to allow assessment of sample homogeneity and technical variability.

3. Results

3.1. Immunophenotypic characterization of Kasumi-1 cell line

Early passage Kasumi-1 cell line was subjected to refined immunophenotypic characterization. As shown in Fig. 1A a high percentage of Kasumi-1 cells stains positive for CD34, CD38, CD33, CD13, and CD4, while a lower percentage is positive for the lineage differentiation markers CD3, CD8, CD16, CD19, CD20, CD14, CD41 and CD235. The CD117 antigen (KIT receptor), tested because of its constitutive activation in Kasumi-1 cells [8], resulted to be expressed in the large majority (83.9%) of the cells. The fraction of $CD34^+CD38^-$ cells, analysed by double staining FACS analysis, was highly represented (48.4%) (Fig. 1B and C). On the opposite, Kasumi-1 cells kept in continuous culture in our laboratory, showed a dramatic decrease in the $CD34^+$ cell subpopulation leading to depletion of the $CD34^+CD38^-$ compartment (till to 0.5%) (data not shown). Thus early passage Kasumi-1 cell line retains the immunophenotypic characteristics of the original cell line established by Asou et al. [10] and presents a high fraction of cells endowed with the $CD34^+CD38^-$ immunophenotype.

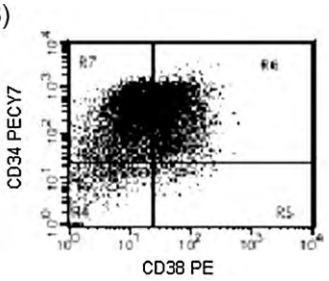
3.2. Kasumi-1 $CD34^+CD38^-$, $CD34^+CD38^+$ and $CD34^-$ subpopulations share AML1/ETO transcript and KIT mutation but differ in KIT expression

The presence of the AML1/ETO transcript in $CD34^-$, $CD34^+CD38^-$ and $CD34^+CD38^+$ subpopulations was monitored by RT-PCR. We detected the presence of the chimeric transcript in all the analysed subpopulations (data not shown). Similarly sequencing of *KIT* exon 17 showed the presence of the N822K mutation in all the selected subpopulations (data not shown). On the contrary a difference in the percentage of KIT (CD117) expressing cells was observed among the above subpopulations

(A)

MARKER	POSITIVE CELLS (%)
CD 34	91
CD 38	54
CD 33	98,7
CD 117	83,9
CD 13	74,5
CD 3	12,47
CD 4	28,8
CD 8	3,8
CD 16	12,45
CD 19	4,25
CD 20	8,5
CD 14	2,26
CD 41	7,5
CD 235	4

(B)



(C)

POPULATION	%
CD34-	6
CD34+CD38-	48,4
CD34+CD38+	46,7
KASUMI-1	100

Fig. 1. Immunophenotypic analysis of early passage Kasumi-1 cell line. (A) Single staining analysis with a panel of selected markers. (B) Representative double staining analysis with anti CD34 and anti CD38 antibodies. (C) Percentage of indicated populations: results are the mean of three independent experiments.

with a higher percentage of positive cells in the $CD34^{+}CD38^{-}$ and $CD34^{+}38^{+}$ compartments in comparison with $CD34^{-}$ cells (Fig. 2A and B).

3.3. Cytogenomic characterization of Kasumi-1 total cell line and selected subpopulations

Characterization of total Kasumi-1 cell line: the first cytogenetic characterization of Kasumi-1 cell line, performed by G banding analysis [10] constituted our main reference.

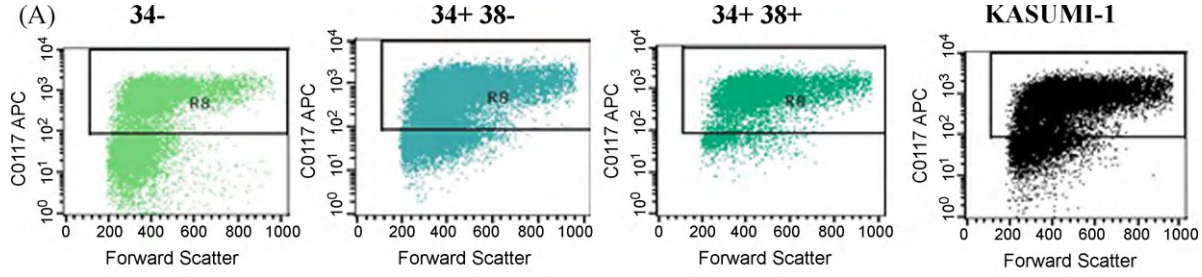
We addressed the cytogenomic analysis of unsorted Kasumi-1 cells by using two different complementary techniques, namely SKY and 244K array-CGH. The results were integrated with the data from conventional cytogenetic analysis (Fig. 3A).

SKY analysis of >50 metaphases showed that DMSZ Kasumi-1 cell line is composed by three cell clones with a 46–48 modal chromosome number, being the chromosome number variation mainly accounted for by the cell-specific number (1–3) of small chromosome 4-derived markers (Fig. 3B). Out of 52 metaphases analysed in the present study, 34 (65%) were found to contain one extra chromosome 4 derived marker, 11 (21%) two copies, while three (5.78%) contained up to 3 markers.

SKY analysis confirmed both the originally reported t(8;21) and Y chromosome loss [10] and the presence of three unbalanced translocations, t(2;8), t(9;15) and t(13;16) all not visible by conventional cytogenetic analysis and subsequently defined as der(2)t(2;8)(q33.3;q24.13), der(9)t(9;15)(p21.2;q21.1) and der(16)t(13;16)(q14.11;p12.1) [13].

SKY analysis also showed trisomy of chromosome 10 (Fig. 3B) in keeping with our QFQ-banding analysis and with the characterization performed by Horsley et al. [13]. *Array-CGH* analysis using an oligonucleotide platform (Agilent, 244K) allowed us to precisely list copy number changes (losses and gains) across the whole Kasumi-1 cell genome. Data can be retrieved in GEO database using accession number GSM506744. As shown in the schematic of Fig. 4A the molecular profiling is more detailed than that defined by Horsley et al. [13] and Rucker et al. [14], with 22 losses present (Fig. 4B) and 12 gains (Fig. 4C). In accordance with the presence of chromosome 4 derivative markers observed by SKY and previous FISH experiments showing that the minute marker chromosomes are derived from duplicated *KIT* containing 4q11 region [8,15], a 14.68 Mb gain of the pericentromeric region of chromosome 4 was found and precisely mapped to 4p12-q13. The genomic representation level of this region, expressed in a log 2 scale is 1.05 that corresponds to a ratio Kasumi-1 alleles/reference DNA alleles close to 2, indicating

(A)



(B)

POPULATION	CD117 POSITIVE CELLS (%)
CD34-	64,5
CD34+CD38-	85,1
CD34+CD38+	93
KASUMI-1	83,9

Fig. 2. (A) Dot plots of CD117 expression in Kasumi-1 selected populations. (B) Percentage of CD117 positive cells in the indicated subpopulations.

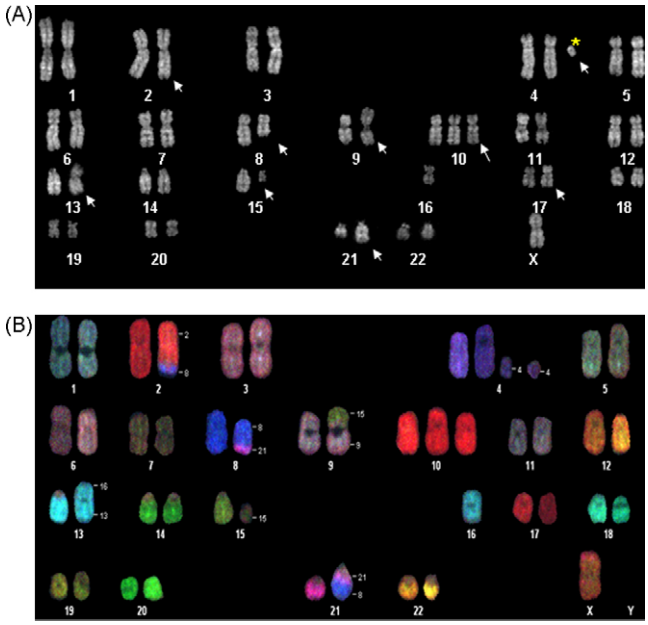
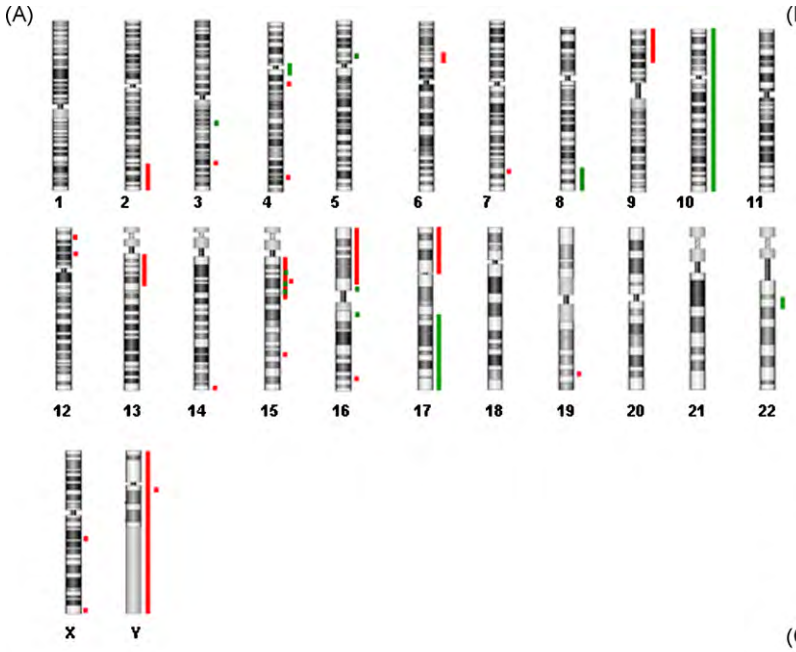


Fig. 3. Representative metaphases of the Kasumi-1 cell line, after (A) conventional QFQ-banding and (B) SKY analysis. Arrows point to chromosomal translocations and trisomy 10, while the stars indicate the chromosome 4-derived markers.

that the region is duplicated. The complete list of genes included in this region is reported in Fig. 5A: besides *KIT* many other potential genes responsible for the "second" hit of leukemogenesis, *CHIC2*, *PDGRA*, *KDR* are here mapped. Another documented gain region is the 22.38 Mb 8q24-qter region including 141 genes, among which well known *MYC* and the regulator of MAP kinase, *TRIB1*.

Cytogenomic characterization of Kasumi-1 subpopulations: next we evaluated whether any difference in copy number sequences across the genome was detectable between $CD34^+CD38^-$ and $CD34^-$ Kasumi-1 subpopulations. QFQ banding of FACS-sorted populations did not show any significant difference (data not shown). Array-CGH performed on DNA from both $CD34^-$ and $CD34^+CD38^-$ cells evidenced all the main gains and losses identified in Kasumi-1 unsorted cell line. In particular the 4p12-q13.1 *KIT* containing region was found over-represented at the same extent in both subpopulations indicating that the lower expression of CD117 (KIT) in the $CD34^-$ subpopulation is not imputable to a different dosage of the *KIT* locus (Fig. 5B). However four genomic regions were found by array-CGH to be differentially represented: the number of oligonucleotides responsible for the signal together with the mapped genes are reported in Table 1 $CD34^+CD38^-$ cells lack the chr3q13.2-q13.31 and chr22 q11.23 gain regions which are overepresented in $CD34^-$ cells. A single gene resides in the 38 Kb 22q11.23: *GSTT1* (glutathione S-transferase theta), a member of the GSTs family of enzymes. Moreover $CD34^+CD38^-$ cells, differently



(B)

CHR	DELETION
chr2	q33.2 - q37.3
chr3	q26.1*
chr4	q13.2*
chr4	q34.1*
chr6	p21.32*
chr7	q34*
chr9	p24.3 - p21.1
chr12	p13.31*
chr12	p11.21*
chr13	q11 - q14.11
chr14	q32.33*
chr15	q11.2 - q21.1
chr15	q14*
chr15	q25.1
chr16	p13.3 - p11.2
chr16	q23.1*
chr17	p13.3 - p11.1
chr19	q13.41*
chrX	q21.1
chrX	q28*
chrY	p11.31-q11.223
chrY	q11.21*

(C)

CHR	GAIN
chr3	q13.2 - q13.31
chr4	p12 - q13.1
chr5	p13.2
chr8	q24.13 - q24.3
chr10	p15.3 - q26.3
chr15	q13.1
chr15	q14
chr15	q15.1
chr16	p11.2
chr16	q12.1
chr17	q21.32 - q25.3
chr22	q11.23*

Fig. 4. (A) array-CGH on the Kasumi-1 cell line. The ideogram reports regions of gain in green and regions of loss in red. The panels on the right report deleted (B) and amplified/duplicated (C) genomic regions.

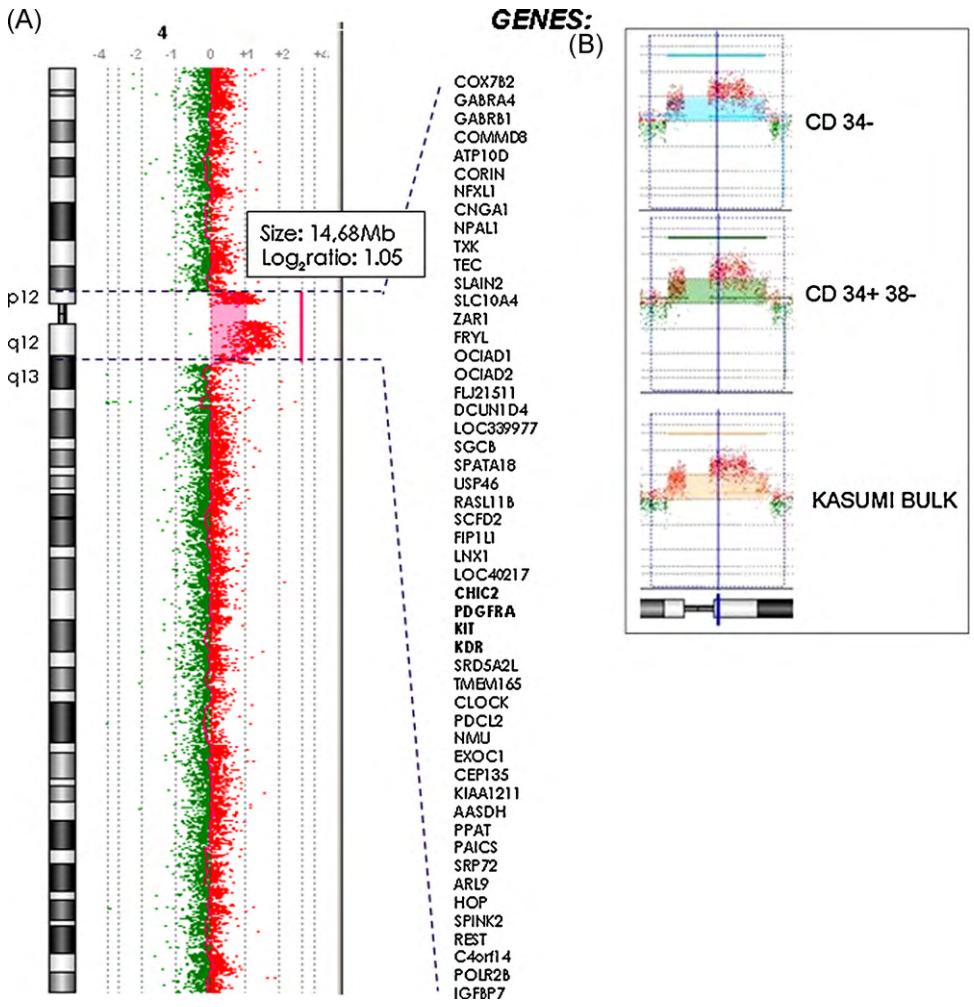


Fig. 5. Genomic amplification at chr4p12-q13.1 and gene content in Kasumi-1 cell line (A). Detail of chr4 p12-q13.1 region in CD34⁻, CD34⁺CD38⁻ and unsorted Kasumi-1 cells (B).

from Kasumi-1 unsorted cells and CD34⁻ fraction do not have the loss of chr12 p11.21, containing *BICD1*, a gene regulating telomere length in humans [16]. Array data about CD34⁻ and CD34⁺CD38⁻ cells can be retrieved in GEO database using the accession numbers GSM506867 and GSM506973, respectively.

3.4. Analysis of miRNA in Kasumi-1 and its FACS-sorted subpopulations

We analysed the expression pattern of 723 human miRNAs from two different series of bulk Kasumi-1 cell line and its CD34⁻, CD34⁺CD38⁻ and CD34⁺CD38⁺ subpopulations using a miRNA microarray platform (Agilent Technologies). Unsupervised analysis (data not shown) evidenced a global expression profile shared by Kasumi-1 and its subpopulations, but a set of 28 miRNAs was found to be differentially expressed (ANOVA, $p < 0.05$) across all samples, as stated by SNK post hoc test (Table 2), and were used for subgroups classification (Fig. 6). All data can be retrieved in ArrayExpress database using the accession number E-TABM-909. Given our interest in CD34⁺CD38⁻ cells we focused on a few miRNAs differentially expressed in this subpopulation. As shown in Fig. 6 only miR-584 and miR-182 appear to be significantly over-

Table 1
Genomic regions differentially represented in Kasumi-1 CD34⁺CD38⁻ and CD34⁻ subpopulations. Losses are indicated in red, gains in green. *n* indicates the number of oligonucleotides involved in the gain/loss. Asterisks indicate CNVs.

	CD34-		CD34+CD38-		
CHR AND CYTOBAND	n.	log2 pvalue	n.	log2 pvalue	GENES
Chr 12 p11.21*	17	-1,08	//	//	BICD1
Chr 14 q21.3	18	-0,94	//	//	POLE2, KLHDC1
Chr 22 q11.23*	7	1,54	//	//	GSTT1
Chr 3 q13.2 -q13.31	139	0,55	//	//	BOC, WDR52, CCDC52, SIDT1 ATP 6V1A, GRAMD1C, ZDHHC23 KIAA1407, QTRTD1,DRD3, ZNF80 NAT13, TIGIT, ZBTB20, KIAA2018

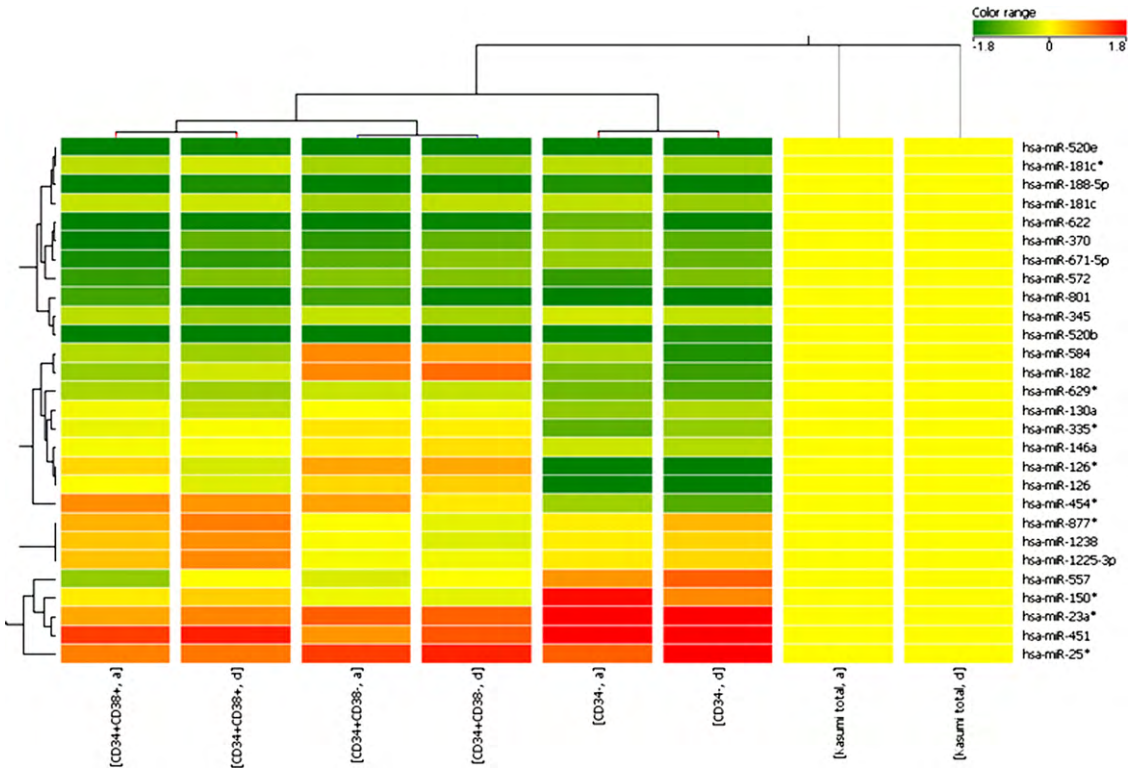


Fig. 6. Expression profiling of the 28 miRNAs differentially expressed among Kasumi and its FACS-sorted subpopulations: the relative value for each sample is represented by color graded from red (high expression) to green (low expression).

Table 2
miRNAs expression at comparison among Kasumi-1 and its different subpopulations. Entities found to be differentially expressed are represented in the blue boxes, while entities found not to be differentially expressed are represented in orange boxes.

GROUP NAME	KASUMI-1	CD34+CD38-	CD34-	CD34+CD38+
KASUMI-1	28	17	24	16
CD34+CD38-	11	28	12	8
CD34-	4	16	28	14
CD34+CD38+	12	20	14	28

expressed in the $CD34^+CD38^-$ fraction in comparison with both $CD34^-$ and $CD34^+CD38^+$. The increased expression of miR-584 and miR-182 in the $CD34^+CD38^-$ cell fraction as compared to Kasumi-1 cell line and $CD34^-$ and $CD34^+CD38^-$ subpopulations has been validated by quantitative expression analysis (Fig. 7).

We used different bioinformatic tools to identify putative target genes predicted by at least two algorithms among TargetScanS, Miranda and PicTar (see Section 4).

4. Discussion

We report on the comparative cytogenomic, immunophenotypic and miRNAs expression profile analysis of the AML Kasumi-1 cell line and its three FACS-sorted subpopulations, aimed at disclosing peculiar differences marking the fraction with the $CD34^+CD38^-$ immunophenotype, where Leukaemia Initiating Cells reside, according to a large body of previous studies [17].

The overall characterization showed that early passage Kasumi-1 cells retain the main immunophenotypic and cytogenetic features of the original cell line. In our hands a dramatic drop in the expression of the CD34 stem cell marker has been observed in cells maintained in culture for long time under standard conditions. This finding raises some caution on the use of cell lines extensively kept in culture and encourages to retype them for markers at focus in the study. Use of early passage Kasumi-1 cell line allowed us to avoid this pitfall providing also the advantages of an unlimited source of homogeneous material for cell sorting, which would have been difficult with primary AML samples. The detailed cytogenomic analysis, integrating QFQ banding, array-CGH and SKY methods, allowed us to refine the previously reported molecular profiling [13,14]. Both the t(8;21) and the loss of the entire Y chromosome

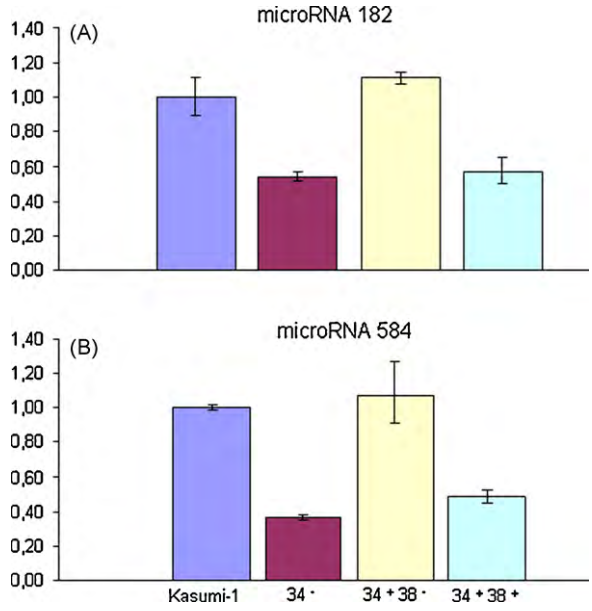


Fig. 7. Real Time PCR analysis of miR-182 (A) and miR-584 (B) performed on Kasumi-1 and its subpopulations (34^-, 34^+38^-, 34^+38^+). RNU48 was used as endogenous normalization control.

have been confirmed in all the analysed metaphases together with the three cytogenetically cryptic translocations t(9;15), t(13;16) and t(2;8) revealed by SKY and leading to imbalances shown by array-CGH. As already noted [13] the t(9;15) and t(13;16) are consistent with the originally ascertained absence of the normal chromosomes 9, 13, 15 and 16 and the presence of a der(9) and chromosomal markers of unspecified origin [10], so that we can not exclude their presence in the original cell line. As to t(2;8) it is not possible to assess whether it was present in the original cell line or it represents an alteration superimposed to t(8;21) which has been introduced during in vitro culture. Trisomy of chromosome 4 was originally described in 11 out of 25 metaphases of the patient from whom the cell line was established [10]. Further analysis on Kasumi-1 cells kept in culture showed the loss of the extra chromosome 4 together with the presence of chromosome 4-derived minute markers where the *KIT* gene was mapped by FISH analysis [8]. The presence of the small 4q11-derived marker in about 92% of Kasumi-1 cells, with two and three copies in consistent subsets of cells, indicates that it might confer a proliferative advantage at least in vitro. Interestingly trisomy 4 has been proposed to identify a distinctive subtype of t(8;21) AML [18] which may predict unfavourable evolution, as attested in both pediatric and adult AML cases carrying an increased dosage of a concomitant *KIT* mutation [19–22]. Immediately adjacent to *KIT* in the chromosome 4 gain region is *KDR*/*VEGFR2*. *VEGFR2* has been shown to play an important role in normal hematopoiesis and in the proliferation and survival of haematological malignancies [23,24]. In Kasumi-1 cells it is possible that an increased expression of *VEGFR2* via chromosome 4 derivative markers might be in part responsible for VEGFR2-dependent growth advantage. Consistent with this hypothesis Kasumi-1 cells have been shown to be growth inhibited by VEGFR2 inhibitor and anti VEGFR monoclonal antibodies [25,26].

The overrepresentation in Kasumi-1 cells of the chromosomal segment from 8q24 leading to overexpression of *MYC* and the close *TRIB1* gene is also a common finding in AML [27]. Conversely, a newly intervened alteration, which may have been favourably selected, is trisomy of chromosome 10, observed by M-FISH and BAC microarray CGH in the DSMZ Kasumi-1 cell line [13] and also reported as partial 10q gain in an independent BAC/PAC microarray characterization [14]. Trisomy 10 is an unusual chromosome abnormality in AML, rarely in association with trisomy 4 and is probably linked to a myeloblast subset with $CD7^+$ and $CD33^+$ immunophenotype [28].

Our Kasumi-1 high-resolution analysis showed additional gain and loss regions ranging in size from 38.6 kb to 1270 kb as compared to the previous study performed with BAC microarrays with a resolution of 1 Mb [13]. Altogether our array results overlap with those of Kasumi cell line performed by Affymetrix SNP 6.0, all available through the Cancer Cell Line Project of the Sanger Institute (http://www.sanger.ac.uk/genetics/CGP/CellLine/).

However we missed same subtle copy number alterations because of the lower resolution of our array and the applied stringent criteria as we only included aberrations detected by at least two adjacent oligos with coherent values of gain or deletion. The main differences between the two datasets are due to the polymorphic nature in the copy number of the regions asterisked in tables of Fig. 4B and C (CNVs, http://projects.tcag.ca/variation/), that is related to their different representation in the DNAs used as control in the experiments. Only three subtle aberrations not corresponding to CNVs, a small loss at Xq21.1 and two gains at 5p13.2 and 15q14, were detected by our array and not reported in the Affymetrix SNP 6.0 analysis. These small differences reflect the dynamic genome of cell lines and the likely characterization of different subclones as also attested by the lack of the *KIT* mutation in the Sanger cell line.

Advances in molecular profiling of a cell line like Kasumi-1 which is widely used as model system to test drug sensitivity and interference are instrumental to interpret exactly its biochemical response to multiple assays.

Comparative analysis of Kasumi-1 cell line and its FACS-sorted subpopulations confirmed they all share the distinctive markers represented by AML1/ETO transcript and N822K *KIT* mutation. Conversely, we observed an increased expression of KIT in the $CD34^+CD38^-$ and $CD34^+CD38^+$ cells as compared to $CD34^-$ cells (Fig. 2). CD34 is commonly expressed in all types of childhood leukaemias whereas CD117 is more specific in leukaemia cells committed to myeloid lineage [29]. The presence of CD34/CD117 co-expressing blasts suggests a differential diagnosis of AML [29,30] with an increase in CD34 and CD117 positivity of the bone marrow blast found associated with progression from Myelodysplastic Syndrome to AML [31].

High-resolution array-CGH (Table 1) showed a few differentially represented regions in Kasumi-1 subpopulations. In particular the $CD34^+CD38^-$ compartment lacks the chr 22 q11.23 interval of gain which is present in $CD34^-$ cells. In this region maps only the *GSTT1* gene, which deletions have been associated with an increased risk of de novo AML [32], higher cumulative incidence of relapse and lower event free survival [33] and may influence treatment outcome after chemotherapy [34]. Glutathione S-transferases (GSTs) are a family of enzymes involved in the detoxification of different compounds. A deficit in GSTT1 activity would result in impaired detoxification of environmental genotoxic agents and chemotherapeutic agents leading to an increased risk of developing primary and secondary cancer and treatment related complications [32]. The log *p*-value of region 22q11.23 is close to 1.5 in $CD34^-$ subpopulation, corresponding to a 3-fold increase. Given that $CD34^+CD38^-$cells do not have such gain they might have a lower GSTT1 activity possibly leading to decreased cell detoxification of genotoxic and chemotherapeutic agents. Another opposite difference concerns the balanced representation in the $CD34^+CD38^-$ fraction of the 12p11.2 small interval which is lost in the total cell line and in the $CD34^-$ subpopulation. It is tempting to speculate that maintenance of the dosage of the *BICD1* gene which contributes to telomere length variation in humans [16] may correlate with the stem-like features of the cell compartment where leukaemia cells with stem cell properties reside. Despite these mentioned regions are listed among CNVs, the differences in the genomic content at these loci between the $CD34^-$ and $CD34^+CD38^-$ subpopulations (Table 1) should bear some significance, as the DNAs used as control reference in the experiment were exactly the same. Indeed, independently of the absolute copy number in the subpopulations and in the normal references, the log2 ratio values have to be considered in the comparisons between $CD34^-$ and $CD34^+CD38^-$ and reflect any differences in their respective genomic content. As copy number variations (CNVs) may contribute to the susceptibility to disease, including different cancer types [35], we believe that these observations may have some relevance. Further investigations are required to test the differential representation of CNVs in $CD34^+CD38^-$ compartment of AML cell lines and primary cells.

In order to better characterize Kasumi-1 cell line and its FACS-sorted subpopulations we interrogated a human miRNA platform which represents 723 miRNAs. The expression profile evidenced in particular two miRNAs (miR-584 and miR-182) which are over expressed in the $CD34^+CD38^-$ compartment, a finding also confirmed by quantitative expression analysis. No significant comparison is applicable to miR-584 which is mentioned in a single study on mesothelioma [36] where it was found to be highly expressed. As regards miR-182, several studies on different solid tumors reported its upregulation [37–39] while the only study in AML showed downregulation [40]. However the authors of this work used normal bone marrow as reference while we referred the

miRNA expression profiling of the different Kasumi-1 cell fractions to that of the bulk cell line, making questionable this discrepancy. By bioinformatic tools we searched for putative targets of these two miRNAs focusing in particular on genes involved in cell proliferation and hematopoietic cell differentiation, speculating about their role in the development of AML. One of the targets of miR-584 is CD38, thus confirming the accuracy of our experimental setting. Other putative targets of miR-584 are ADRA1A (Alpha-1A adrenergic receptor) and SSTR4 (somatostatin receptor type 4), genes involved in negative regulation of cell proliferation, HHEX (hematopoietically expressed homeobox protein) that seems to have a role in hematopoietic differentiation [41], FZD7 (Frizzled-7) [42] and MTBP (Mdm2-binding protein) [43] that are involved in WNT and p53 pathways, respectively.

The other miRNA (miR-182) found over expressed in the $CD34^+CD38^-$ subpopulation, has been found involved in breast cancer and the *FOXO1* tumor suppressor gene has been validated as target raising a possible role of this miRNA in AML development [37]. Another putative target is NPM1 (Nucleophosmin), a gene encoding for a negative regulator of cell proliferation, whose role in AML has been extensively investigated [44]. Other predicted targets of miR-182 are PYGO2 (Pygopus homolog 2) [45], a WNT pathway gene and SPR-2 (Transcription factor Sp3), a transcriptional factor with a possible role in hematopoietic differentiation [46]. Functional studies are needed in order to validate these predicted targets.

In conclusion, the overall cytogenomic and miRNA profile of Kasumi-1 cell line and its selected subpopulations, in particular the $CD34^+CD38^-$ one, showed a few differences peculiar of this fraction. Since Kasumi-1 cell line is a faithful model of t(8;21) AML, these findings may be of relevance to studies addressing the precise characterization of the compartment endowed with stem cell-like properties in this AML subtype.

Conflict of interest

The authors declare that there is no conflict of interests.

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