UNIVERSITY OF INSUBRIA

XXIX Cycle of Ph.D. program in

Experimental and Translational Medicine

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"Genomic aberrations in Acute Myeloid Leukemia and significance of constitutional Copy Number Variations in the development of Post-Transplant Lymphoproliferative Disorders"

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1 - INTRODUCTION

1.1 Genetic landscape of tumors

The majority of human cancers arise from cells that are unable to maintain genomic stability: cells must accumulate a significant number of genetic changes in order to progress to cancer.
Even if genomic instability is not a requirement, carcinogenesis may proceed more quickly when the genome is unstable, especially if the number of changes required for progression is large and the genomic instability arises early (Beckman RA et al., 2006).
Tumors demonstrate several mechanisms of genomic instability, that affect the genome at different levels, ranging from a single nucleotide changes to chromosome rearrangements, and the gain and loss of chromosome arms or aneuploidies or cryptic Copy Number Variations (CNVs).

Neoplasms are characterized by Structural / Numerical, balanced/unbalanced chromosomal abnormalities, that often can coexist in complex karyotypes (Mietelman database).
Not all seemingly balanced translocations turn out to be exactly balanced when analyzed at the base pair level. Deletions, inversions, and duplications in, and around, the translocation breakpoints may accompany what looks cytogenetically as balanced changes, both in malignant diseases and in constitutional translocations (Howart KD et al., 2011).

In the genome there are also mutations involving a change in a single base pair, called Point Mutations that may produce Missense mutations, Nonsense mutations and Frameshift mutations and small deletions of a few base pairs.
Each of these conditions may have a drastic effects on phenotypic cells expression and may be responsible for tumorigenesis.

1.1.2 Classical cytogenetics, FISH and array CGH analysis in hematological neoplasms

Classical cytogenetic analysis plays a pivotal role in the diagnosis, prognosis, identification of the genes involved in the development of hematological malignancies and also influences the decision about the treatment of the disease (Kayser S et al., 2012).
It is cost-effective and it is considered the gold standard in the diagnostic routine work-up of leukemias.

On the other hand, conventional karyotype has the drawbacks of poor chromosome quality and limited resolution.
The generally accepted banding resolution for a routine bone marrow karyotype is 300-400 bands per haploid chromosome set (Gorczyca W et al., 2008).

Fluorescence *in situ* hybridization (FISH) expands cytogenetics by identifying cryptic submicroscopic chromosome rearrangements that are not detectable by routine karyotyping.
FISH analysis is highly dependent on clinical suspicion for the "choice" of the locus to analyze, especially in hematological cancers, where it can identify losses/gains of little regions cointaining onco-genes or the presence of fusion genes.

In addition to the majority of abnormalities identifiable by classical cytogenetic and FISH, the advent of high resolution genome assays, including **Comparative Genomic Hybridization (CGH)** has enabled the detection of clinically significant submicroscopic Copy Number Variations (CNVs).

Comparative genomic hybridization array is a high-throughput genomic screening that is able to detect genomic aberrations in order to redefine karyotype results and to discover submicroscopic or cryptic rearrangements possibly involving onco-genes.
Microarray analysis also eliminates the need of using dividing cells and can be performed on uncultured speciments to provide a more accurate assesment of abnormalities (Dougherty MJ et al., 2011).
For these reasons, array−based platforms are now widely used in routine diagnosis, especially for constitutional diseases, and are gaining more and more popularity in assessing hematological cancer samples.

1.1.3 Copy Number Variations and Loss of Heterozygosity

In 2004 two landmark studies demonstrated that genomic submicroscopic variations are widespread in normal human genome (Iafrate et al., 2004; Sebat et al., 2004). Several analysis of this type of genomic variability has followed, revealing the role of DNA CNVs as an essential contributor to individual variability.

CNVs are a form of structural variations, defined as genomic segments of at least 1kb in size, present in a variable copy number (Shlien A et al., 2009).
CNVs alter the number of included genes or can alter the trascription of genes by disrupting the start/end point, proximal or distant regulatory regions.

A direct relationship between CNVs and the expression of the genes encompassed by such CNVs has been extensively documented both in constitutional diseases and in tumors.

Although common CNVs may contain cancer-related genes and contribute to carcinogenesis, only the very rare CNVs (less than 1% in the population) are likely to have high penetrance for cancer and hematological malignancy. The role of a given CNV in cancer development depends not only on its gene content, but also on its interaction with other genomic variants and with environmental triggering agents (Krepischi et al., 2014).

We must consider that not all CNVs are causative of disease or correlated to cancer development; many genomic imbalances are polymorphic, because commonly present in the healthy population. Polymorphism is defined as a genomic variant present in at least 1% of healty population. Polymorphism can modify the gene dosage or they may not have relevant clinical impact if the region involved in the deletion/amplification is gene-free.
In constitutional diseases, the common guidelines for interpretation of the possible phenotypic impact of CNVs include comparison of found CNVs with CNVs in health individuals, stored on the Database of Genomic Variant (DGV db).
Such polymorphic region, excluding the very common ones, must be taken into account in oncology because of their hypotetic predisposing significance.

Loss of heterozygosity (LOH) is detectable in many forms of cancer, including leukemia and lymphomas and it contributes to tumorigenesis through the loss of one allele of tumor suppressor gene.
Genetic mechanisms leading to LOH are highly variegated. It can be caused by deletion, which can involve whole chromosome or a smaller region, by mitotic non-disjunction or by mitotic recombination between two homologous chromosomes.

LOH may be due to two kinds of events: deletions or Uniparental Disomy (UPD).
Deletion is a loss of chromosomal region and LOH is the obvious consequence.

UPD refers to the situation in which both copies of a chromosome pair have originated from the same parent (Robinson, W.P et al., 2000).

UPD can occur during gametogenesis or during early somatic cell divisions in the zygote; in these cases UPD is a constitutional condition.
UPD can also occur as a consequence of somatic recombination during mitotic cell divisions (mitotic or somatic recombination) and thus, can be an important step in cancer development, as well as a contributing factor to other late onset diseases.

The best choise tecnique to identify both CNVs and LOH is high resolution single nucleotide polymorphism genomic microarray.

1.2 Clinic overview of Hematologic Neoplasm

Blood cancer or leukemia is a general term used to describe a group of heterogeneous neoplastic disorders affecting white blood cells.

The main features of this disease is an uncontrolled proliferation of hematopoietic cells that, in the most of the cases, do not retain the physiological capacity to differentiate normally into mature blood cells.

This differentiation arrest can occur in every maturation stage and in every cell lineage (myeloid / lymphoid) of blood cell differentiation, resulting in distinct forms of leukemia.

Over the years several attempts have been made to devise a classification system for the different types of leukemia. Based on the patient survival rate and on the degree of maturation of the cells, the leukemias are classified as acute or chronic, which are further classified into lymphoid or myeloid types depending on the cell lineage involved in the disease (Castoldi G et al., 2007)

There are four general types of leukemia, namely Acute Lymphoblastic Leukemia (ALL), Acute Myeloid Leukemia (AML), Chronic Lymphocytic Leukemia (CLL) and Chronic Myeloid Leukemia (CML) (Table 1).

CLL and AML are the most common form of leukemias in adults.

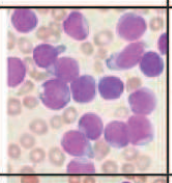
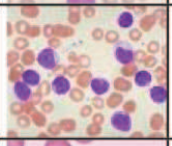
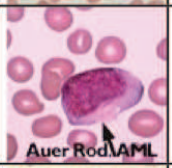
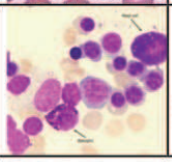
condition	picture	etiology	cell involved	morphology	clinical presentation	CBC results	demographic
acute lymphocytic leukemia (ALL)		chromosomal abberation resulting in abnomal transcription factors that affect development of B and T cells	immature B or T cell (marrow)	condensed chromatin, scant cytoplasm, small nucleoli	stormy onset, symptoms related to depressed marrow function, bone pain, CNS manifestations	anemia, thrombocytopenia, variable WBC's, **>30% lymphoblasts**	children.
chronic lymphocytic leukemia (CLL)		chromosomal deletion or possible somatic hypermutation of postgerminal or naïve B cells	peripheral B or T cell (lymph nodes)	**smudge cells,** condensed chromatin, scant cytoplasm	asymptomatic or nonspecific, LAD, hepatosplenomegaly,	sustained abs. lymphocytosis >5000/uL, low platelets in 20-30%	most common leukemia in adults. twice as common in men.
acute myelogenous leukemia (AML)		oncogenic mutations impede differentiation, accumulating immature myeloid blasts in marrow	immature myeloid lineage cells (marrow)	**auer rods** (abnormal lysosomes), myeloblasts, monoblast	anemia symptoms, spontaneous bleeding, petechiae and ecchymoses	anemia, neutropenia, thrombocytopenia, **>30% myeloblasts**, auer rods	adults.
chronic myeloid leukemia (CML)		tyrosine kinase pathway related chromosomal translocation-philadelphia chromosome	pluripotent hematopoetic stem cell (marrow)	hypercellular marrow, elevated eosinophils and basophils	insidious onset, mild anemic symptoms, splenomegaly	asx WBC> 50,000, symptomatic **WBC>200,000-1,000,000,** some blast forms, **increased eosinophils and basophils**	ages 20-50, rare in children.

Table 2 - Types of Leukaemia

The two most popular classification systems presently in use worldwide are the French–American–British (FAB) classification and the World Health Organization (WHO) classification.
The first was established in 1976 and it is based on the morphological and cyto-chemical characteristics of the leukemic cells, the second was estabilished in 2001 and it has been recently revised.

In 2016 The World Health Organization (WHO) published and reviewed a classification system for tumors and leukemias incorporating immunophenotypic and clinical information to define a clinically and biologically relevant disease nomenclature. Genetic investigation is essential part of the diagnostic and prognostic field.

1.3 Acute Myeloid Leukemia (AML)

Acute myeloid leukemia (AML) is defined as a clonal disorder caused by malignant transformation of a bone marrow–derived, self-renewing stem cell or progenitor, which demonstrates a decreased rate of self-destruction as well as aberrant, and usually limited, differentiation capacity. These events lead to an increased accumulation of these malignant myeloid cells in the bone marrow and other organs by these malignant myeloid cells.
AML is nowadays considered a disease of the elderly, based on the median age at the diagnosis (67 years) and it is also slightly more common in males than in females.
The presenting symptoms of AML are remarkably variable, but pallor and redness resulting from anemia, easy bruising/spontaneous bleeding due to thrombocytopenia, fever/ infections caused by the lack of normal leukocytes are often present.

The FAB classification system represents the first attempt to distinguish between the different types of AML and it defines eight subtypes (M0 to M7).
According to the WHO classification, the current major subtypes of myeloid neoplasms and acute leukemias are: AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, Therapy-related myeloid neoplasms, AML not otherwise specified NOS, myeloid sarcoma, Myeloid proliferations related to Down Syndrome, Blastic plasmacytoid dendritic cell neoplasm and Acute leukemias of ambiguous lineage.
The lastest update of the WHO classification provides few changes to the existing disease categories and introduces a new category called “Myeloid neoplasm with germline predisposition” (Dohner et al., 2016).

AML can arise *de novo* or secondarily either due to the progression of other diseases, like Myelodisplastic syndrome. Up to 15% of patients affected by AML develop the disorder after treatment with cytotoxic chemotherapy.

Development of AML has also been correlated to the exposure to a variety of environmental agents, with a strong correlation between exposure history and cytogenetic abnormalities. Radiation, benzene inhalation, alcohol use, smoking, dyes, and herbicide and pesticide exposure have been all included as potential risk factors for the development of AML (West RR et al., 2000; Crane et al., 1996)

1.3.1. Chromosomal aberrations in AML

Non-random chromosomal aberrations, which often result in gene arrangements, can be detected in approximately 50% of newly diagnosed AML patients. According to SIGU (Italian Society of Human Genetics) and international guidelines, chromosomal aberration in AML must be clonal and thus present in at least 3 cells (in case of monosomies) or in at least 2 cells (in case of trisomies or structural rearrangements).
Actually cytogenetics abnormalities are an important diagnostic and prognostic factor in predicting remission rate, relapse, and overall survival of AML patients (Martens JHA et al., 2010; Dohner et al., 2015).

Several chromosomal abnormalities such as monosomies or partial/entire deletions of chromosomes are common in AML (Byrd et al., 2002). The most common abnormalities in AML are:

- Losses affect mostly chromosome 5 and 7 (-5/5q-, -7/7q-); -17/17p-, 18/18q-, 12p-, -16/16q- are also frequently found.
- Gains generally affect 8/8q, (+8/+8q), +21/+21q, +11q, +22q (Rucker et al., 2006).

All the chromosomal aberrations in leukemia are reported in the Mitelman database (Mitelman database of chromosome aberrations and gene fusion in cancer. Last update November 2016).

The most commonly identified abnormalities are reciprocal translocations, which create a fusion gene encoding a chimeric protein. The most frequent chromosomal aberrations and their corresponding fusion genes are shown below.

Translocations	Oncofusion protein
• t(8;21)(q22;q22)	AML1-ETO
• t(15;17)(q22;q21)	PML-RARα
• inv(16)(p13.1q22) or t(16;16)(p13.1;q22)	CBF□-MYH11
• der(11q23)	MLL-fusions
• t(9;22)(p21.3;q23.3)	BCR-ABL1
• t(6;9)(p23;q34.1)	DEK-CAN
• t(1;22)(p13.3;q13.3)	OTT-MAL
• t(8;16)(p11;p13)	MOZ-CBP
• t(7;11)(p15;p15)	NUP98-HOXA9
• t(12;22)(p13.3;q13.3)	MN1-TEL
• inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)	RPN1-EVI1
• t(16;21)(p11;q22)	FUS-ERG

(Chandra Kumar C et al., 2011; Mrózek K et al., 2009)

1.3.2 Most recurrent and cryptic CNVs in AML

In addition to large chromosomal rearrangements, cryptic genomic changes and molecular changes have also been correlated to the development of AML. There are many studies aimed at detecting recurrent or individual copy number changes.

Losses are generally more frequent than gains.
The most recurrent submicroscopic aberrations reported in literature are listed in table 2 and they are showed in figure 1.

CHROMOSOME	CNV GAIN Cytoband	CNV LOSS Cytoband
1	p36.13 p35.3	p36
2	q33.1	q33.2
3	//	p14.1 q21.1
4	//	q13.1 q22.2 q24
5	p15.33 q13 q35.1	q31 q31.2 q32
6	q23.3	q24.1 q27
7	//	p14.1 p12.2 q11.21 q22 q31 q31.32 q32.3 q35q36
8	q21.2 q23.2 q24.12 q24.13 q24.13q24.21	q23
9		p21.3 p13.2
10		q11.2
11	q12q14 q23.3	q25
12	p13.32	p13.1 p13.1p12.3
13		q14.2 q14.3
14		q31.1q32.11
15	q11.2 q21.3	q21.3
16		q22 q22.1 q23.1q24.3
17	q12	p13.2p13.1 p13.1 p11 q11 q11.2 q24.3
18	//	p11.2 q12.3q21
19	p13.2 p13.1	//
20	//	q13.12 q12q13.1
21	q22.2	q22.12

Table 2 - Most recurrent CNVs in AML (Paulsson K et al., 2006; Walter MJ et al., 2009; Rücker FG et al., 2006; Kjeldsen et al., 2015; Zhang R et al., 2015).

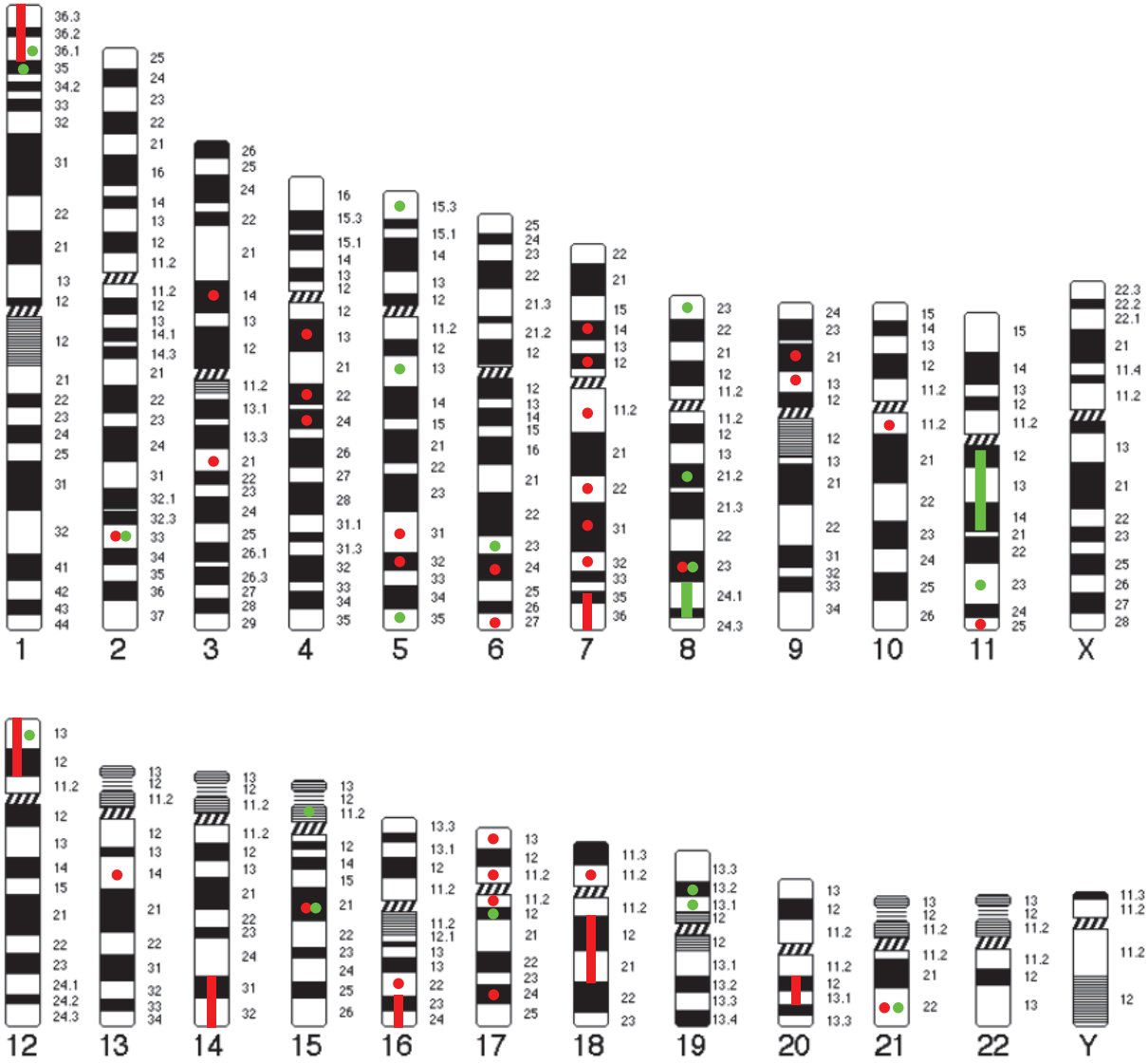


Figure 1 - Ideogram with the most recurrent CNVs in AML. Deletions are represented in Red, Duplications in green (Paulsson K et al., 2006; Walter MJ et al., 2009; Rücker FG et al., 2006; Kjeldsen et al., 2015; Zhang R et al., 2015).

Cytogenetically normal AML accounts for about one-half of total AML (Dohner et al., 2010).

Karyotypically normal AMLs (NK-AML) are prognostically various and generally classified as an intermediate risk; therefore, the ability to identify molecular cytogenetics changes in patients with NK-AML will deepen our understanding of the genetics behind AML and can open the possibility to develop more specific therapies as well as improved patient classification schemes.

To date, only few studies have evaluated CNVs-SNPs in patients with NK-AML.

Tyybakinoja et al., in 2007 investigated Copy Number aberrations in 26 NK-AML patients and he detected novel cryptic aberrations in 22 out of 26 cases. Molecular karyotype showed deletions in 1q41 8q24.11, 12p12.2, 12p13.2, 18q21.32 and duplications in 3p21.3 and 8q24.12q24.21.

Akagi et al., in 2008 found some CNVs in NK-AML: Gains were reported to affect chromosomes 1q43, 18q21.2, 8q24.12-q24.21; found Losses were located on 2p23.1, 2q36.2, 3p26.3, 4q24, 8p23.3, 9p21.3-p21.2, 12p13.31p13.2, 14q21.2, 17q11.2 and 21q21.2.

Bullinger et al., in 2009 studied 157 AML NK-patients with 50K and 500K affymetrics SNP array and observed both SNPs and CNVs. Genomic losses were more frequently identified than gains.
The following bands were reported as recurrent genomic deletions: 3p14.1-p13, 6q27, 8q23.3, 10q11.21, 11q25, 12p13.2, and 15q21.3, that harbor genes FOXP1 and RYBP (3p14.1-p13) RPS6KA2 (6q27), TRPS1 (8q23.3), HNRPF(10q11.21), ETV6 (12p13.2) and RFXDC2 (15q21.3).

Kim et al., in 2012 studied CNVs in 30 AML patients with normal karyotype and the association between the alterations and the response to treatment. He found gain in chromosome 4, 8 and 17, and he observed many losses ecompassing AML related gene, as shown below:

CHROMOSOME	CNV GAIN Cytoband	AML relevant gene
4	q32-2	//
8	p11.1	
17	q21.32	
CHROMOSOME	**CNV LOSS Cytoband**	**AML relevant gene**
1	p36.33p36.32	HES5, PRDM16
	p36.31	TNFRSF25
2	q31	MTX2
4	p16.3	
5	p15.33	TERT
7	p22.3	ABCB8
	p15.2	
	q36.1	
8	p21.3	PTP4A3
	q24.3	
9	q33.3	PBX3
	q34.2q34.3	
10	q26.3	VENTX
11	p15.5	
12	q24.33	
14	q32.33	AKT1, KIAA0284
16	p13.3	ABCA3
	q24.1	
	q24.2q24.3	CBFA2T3, FANCA
17	p13.2	
	q12	MLLT6
	q25.3	CD7
18	q23	
19	p13.3	PRTN3
	p13.11	
	q13.11	CEBPA
	q13.31q13.32	
	q13.42	
	q13.43	
20	q13.33	
21	q22.3	
	q11.1	
	q13.31	
	q13.33	

Spina et al in 2015 studied 36 patients with AML and normal karyotype. CGH array showed no alterations in 22 out of 36 cases and, in the remaining 14 cases, it allowed the detection of 43 imbalances: 24 duplications and 19 deletions, that were not previously identified through CC and/or FISH.

Some of these alterations were highly repetitive, as they were found in more than 3 patients: gain of 19q13.12 (8/14 cases), gain of 7q35 (5/14 cases) and loss of 3p14.2 (2/14 cases).

About 10 years ago genome-wide analysis of single nucleotide polymorphisms in AML patients has revealed that about 20% of them exhibited large regions of homozygosity that could not be detected by visible chromosomal abnormalities in the karyotype.

Some authors applied SNP array in AML cases and identified partial uniparental disomy (pUPD) in a relevant percentage of cases.

The most recurrent sites of loss of heterozygosity found in AML patients affected chromosomes 11p, 11q, 13q (Raghavan et al., 2005; Gupta et al., 2008; Gronseth et al., 2015).
Additional recurrent abnormalities were located on 2p, 2q, 11p, 11q, 17p, 17q, 21q and Xq loci (Raghavan et al., 2005; Gronseth et al., 2015).

More recently, Bullinger and colleagues used 50K and 500K Affymetrix SNP arrays to examine 157 cases of cytogenetically normal AML.
The cohort showed that 12% UPDs most commonly involved the chromosomal regions 6p, 11p, and 13q; all UPDs were >29 Mb.
The UPDs were associated with mutations in NPM1 or CEBPA, which suggested, according to the findings of Fitzgibbon and colleagues, that UPDs may affect genes that are critical for hematopoiesis (Bullinger et al., 2009; Fitzgibbon et al., 2005).

All these studies confirmed the clinical relevance as well as prognostic significance of CNV/SNP lesions in NK- AML, which would allow a better prognostic stratification of AML patients and more appropriate treatments of the disease.

1.3.3 Genic mutation in AML

During the last decade, several studies have shown that the presence of specific gene mutations and/or changes in gene expression can lead to further classify AML cases and can have an effect on the patients' prognosis. Key molecular abnormalities have been identified and are now used to predict outcomes; moreover, they help to guide the treatment of AML patients (Lindsley RC et al., 2015; The Cancer Genome Atlas Research Network, 2013).

Genetic mutations in AML are classified into two main categories: Class I and II. The first results in the activation of pro-proliferative pathways and survival, the second impairs normal hematopoietic differentiation for leukemia development and apoptosis (Kavianpour et al., 2016).

Class I mutations involve gene such as FLT3, K/NRAS, TP53 and c-KIT, which are found in

approximately 28, 12, 8 and 4% of cases, respectively.
Class II include NPM1 and CEBPA mutations, which are found in approximately 27% and 6% of cases, respectively.

Alterations in genes involved in epigenetic regulation have recently emerged as a third class of mutations, with downstream effects on both cellular differentiation and proliferation.

These include mutations in the DNA-methylation related genes DNMT3A, TET2, and IDH-1 and IDH-2, which are found in more than 40% of AML cases (I De Kouchkovsky and M Abdul-Hay 2016)

Patients are generally grouped into three risk categories (Favorable, Intermediate, Adverse), based on both cytogenetic and molecular aberrations (Figure 2).

Risk Category[b]	Genetic Abnormality
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD[low(c)] Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD[high(c)] Wild type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD[low(c)] (w/o adverse-risk genetic lesions) t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*[d] Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214* t(v;11q23.3); *KMT2A* rearranged t(9;22)(q34.1;q11.2); *BCR-ABL1* inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)* -5 or del(5q); -7; -17/abn(17p) Complex karyotype,[e] monosomal karyotype[f] Wild type *NPM1* and *FLT3*-ITD[high(c)] Mutated *RUNX1*[g] Mutated *ASXL1*[g] Mutated *TP53*[h]

[a] Frequencies, response rates and outcome measures should be reported by risk category, and, if sufficient numbers are available, by specific genetic lesions indicated.

[b] Prognostic impact of a marker is treatment-dependent and may change with new therapies.

[c] Low, low allelic ratio (<0.5); high, high allelic ratio (≥0.5); semi-quantitative assessment of *FLT3*-ITD allelic ratio (using DNA fragment analysis) is determined as ratio of the area under the curve (AUC) "*FLT3*-ITD" divided by AUC "*FLT3*-wild type"; recent studies indicate that acute myeloid leukemia with *NPM1* mutation and *FLT3*-ITD low allelic ratio may also have a more favorable prognosis and patients should not routinely be assigned to allogeneic hematopoietic-cell transplantation.[57-59,77]

[d] The presence of t(9;11)(p21.3;q23.3) takes precedence over rare, concurrent adverse-risk gene mutations.

[e] Three or more unrelated chromosome abnormalities in the absence of one of the World Health Organization-designated recurring translocations or inversions, i.e., t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23.3), t(6;9), inv(3) or t(3;3); AML with *BCR-ABL1*.

[f] Defined by the presence of one single monosomy (excluding loss of X or Y) in association with at least one additional monosomy or structural chromosome abnormality (excluding core-binding factor AML).[116]

[g] These markers should not be used as an adverse prognostic marker if they co-occur with favorable-risk AML subtypes.

[h] *TP53* mutations are significantly associated with AML with complex and monosomal karyotype.[87,88-89]

Figure 2 - 2017 European LeukemiaNet risk stratification by genetics [a]

1.4 Post-Transplantation Lymphoproliferative Disorders, PTLD

Post-transplantation lymphoproliferative disorders (PTLD) are an aggressive complication of both solid organ transplantation (SOT) and hematopoietic stem cell transplantation (HSCT) in the setting of immunosuppression.
Immunosuppressive induction and maintenance regimens were carried out to prevent organ graft rejection by the recipient's immune system.
On the downside of pharmacological immunosuppression, a decreased immunological surveillance of infections and malignancies is observed.
Pediatric and adolescent patients after transplantation have an increased risk of cancer development, which is estimated to exceed the normal population up to 45-fold, depending on the type of cancer (Engels EA et al., 2011)

The incidence of PTLD largely depends on the type of organ transplanted, the respective intensity of immunosuppression, and the recipient's viral status prior to transplantation.

Primary EBV infection may develop in an EBV-seronegative recipient who receives an allograft from an EBV-seropositive donor, and it is probably recognized the most important risk factor for PTLD development and often occurs within the first year after transplantation.
It is therefore not surprising that in general, PTLD rates are reported to be higher in pediatric transplant recipients than in adult transplant recipients (Jamali FR et al., 2007).

The type of transplant allograft is another relevant risk factor that influence the probability to develop PTLD.
Reported rates of PTLD are higher in heart, heart-lung, and small bowel transplants compared to kidney and liver transplants. This presumably reflects in part the need for more intense immunosuppression to maintain certain types of allografts. The PTLD after Bone Marrow transplantation ranges from 0.5% to 1.0% (Lawrence T et al., 2007).

Other risk factors implicated in PTLD include Hepatitis C, Cytomegalovirus (CMV) and age younger than 10 or older than 60 years (Buda et al., 2000; Cockfield et al., 2001; Opelz G et al., 2004).

The combination of multiple risks factor can increase the risk of PTLD up to 500-fold compared to patients with no risk factors (Walker Rc et al., 1995).

PTLD include a wide range of diseases, from benign hyperplasia to malignant lymphomas that can arise from B cell. More than 80% of these are EBV correlated although there are some cases of non-EBV correlated PTLD. EBV negative PTLD typically appears as a late complication of transplantation with more aggressive features.

The WHO classification clearly identifies PTLD, grouping them into main categories:
Early lesions, polymorphic PTLD, monomorphic PTLD, and classical Hodgkin lymphoma-type PTLD, as presented below:

Early Lesions (5%) Plasmacytic hyperplasia PTLD and Infectious mononucleosis PTLD
Polymorphic (PTLD 15-20%)
Monomorphic PTLD • B-cell neoplasms (>70%) Diffuse large B-cell lymphoma, Burkitt lymphoma, Plasmacytic neoplasm • T-cell neoplasms (<5%) Peripheral T-cell lymphoma NOS, Hepatosplenic T-cell lymphoma
Classical Hodgkin lymphoma-type PTLD (<5%)

Table 3 - the Who classification of PTLD

The most common malignant PTLD subtype are Post-transplant diffuse Large B-cell lymphoma (PT-DLBCL), Burkitt lymphoma (PT-BL) and plasmablastic lymphoma (PT-PBL).

CGH array in PTLD

- **Post-transplant diffuse large B-cell lymphoma (PT-DLBCL)**

Genetic studies have demonstrated that Post transplantation-DLBCL (PT-DLBCL) has some somatic recurrent genomic aberrations such as gains of 8q24 harboring *MYC*, 3q27 harboring *BCL6*, 18q21 harboring *BCL2*, 7q harboring *CDK6* and loss of 17p13, harboring *TP53.*
Moreover, PT-DLBCL presents Gain of chromosome 2p24-25, 5p, 9q22, 12q22 13q32,18q21 and Non-random losses involved 1p36, 4q, 17p13, 17q23q25, Xp (Poirel HA et al., 2005)

EBV+ and EBV- PTLD are rarely distinguished, but some studies have found some differences:

EBV- PT-DLBCL was associated with gains of 3/3q (encoding *FOXP1*) 7p, 7q and 11q24-q25, 18q, and loss of (4q25-q35) 6q23.3/*TNFAIP3* and 9p21/*CDKN2A*. (Rinaldi A et al., 2010; Lenz G et al., 2008, Moscio J et al., 2016)

EBV+ PT-DLBCL frequently carry trisomies of chromosomes 9 and 11 (Djokic M et al., 2006) and gain of 9p24.1, that harbors PDL1, PDL2 and JAK2 (Yoon et al., 2015).
In one study, aberrant MYC was detected in 40% of PT-DLBCL, independently of the EBV status (Cerri M et al., 2004)

	GAIN	**LOSS**
PT-DLBCL	8q24 (MYC) 3q27 (BCL6) 18q21 (BCL2) 7q (CDK6) 5p	17p13 (TP53) 17q 4q Xp

	EBV negative	
	GAIN	**LOSS**
PT-DLBCL + EBV status	7p/q 11q24-q25 3q (FOXP1)	4q25-q35 6q23.3 (TNFAIP3) 9p21 (CDKN2A)
	EBV positive	
	GAIN	**LOSS**
	+9 +11 9p24.1 (PDL1/2 JAK2)	//

Table 4 – Schematic representation of chromosomal aberration in PT-DLBCL patients

- **Burkitt Lymphoma**

The hallmark of BL and also PT-BL with Burkitt morphology is the presence of translocations t(8;14)(q24;q32) involving MYC gene (Djokic M et al., 2006).
Recently an 11q aberration (Gain/Loss) was detected in both MYC-negative and positive high-grade B-cell lymphomas resembling BL without MYC rearrangements, both at morphological as well as the molecular level. (Salaverria I et al., 2014) (Havelange V et al., 2016)

- **Plasmablastic lymphoma PT-PBL**

Very little is known about the molecular-genetic basis that drives PT-PBL. One study showed that up to 47% of EBV+ AIDS-related PBLs are marked by MYC translocations (Delecluse HJ et al., 1997**)** Array-CGH showed that the genomic aberrations pattern of PBL is similar to that found in DLBCL.

In conclusion, non-random chromosomal imbalances are present in PTLD. The open question is the role of such lesions in the development and progression of this PT-induced lymphoproliferative disorders (Porel HA et al, 2005).

2-AIM OF THE STUDY

2.1 AML Patients

The first aim of the present study was to characterize, by array CGH analysis, the presence of cryptic copy number variants (CNVs) and LOH regions through whole-genome oligonucleotide array CGH+SNP analysis in AML patients with apparent normal karyotype; moreover, this study is aimed at identifing the associations of such alterations with biological / clinical features and outcome.

The second aim was to demonstrate the clinical utility of aCGH in patients with different subtypes of AML.

2.2 PTLD Patients

The aim of this study was to investigate by array-CGH 180K, the presence of constitutional CNVs in the patients that developed a post-kidney transplantation lymphoproliferative disorder (PTLD).

The results have been compared with those found in patients that did not develop lymphoproliferative disorders after a renal transplantation.

The hypothesis of a role of constitutional CNVs as predisposition factors for such cancer development has been recently suggested by different authors (Roland P K et al 2010; Adams S et al., 2009) .

Park et al., in his study identified rare germline copy numbers variations overrepresented in five human cancer (brain, ovarian, colon-rectall, renal) and this findings potentially serve as clinical useful markers conferring increased cancer risk.

Krepischi et al., suggested that a duplication Xq27.2 and a deletion of 13q31.2-q31.3 can predispose to pediatric Hodgkin's Lymphoma and Acute Lymphoblastic Leukemia respectively.

Our hypothesis is that as yet unknow germinal CNVs may contribute as predisposition factors to the development of PTLD in post transplanted patients.

3 – METHODS AND MATERIALS

3.1 Patients

Two groups of patients were studied: the first group consisted of 26 de novo AML patients (32-78 years).
Diagnosis of de novo AML was estabilished at the hematology Department of Ospedale di Circolo e Fondazione Macchi - Varese directed by Prof. Francesco Passamonti. Clinical diagnosis was performed according to FAB criteria.
The most common remission induction regimens for AML include a drug called Idarubicin (12mg/mq/die, day 1-3) and Daunorubicin / Cytarabine (100mg/mq/die, day 1-7) This is often called the "3+7" regimen.
Resistant AML is considered when, after the first induction cycle of chemotherapy, more than 5% of blasts are present in the bone marrow.
Consolidation or post-remission chemotherapy often includes 3 or 4 cycles of high-dose Cytarabine.

The second group of patients consisted of 22 kidney-transplanted pediatric patients: part of them developed lymphoproliferative disorders post transplantation. Some patient showed EBV Viral Load (High: more than 1000 copies/ml; Low: less than 1000 copies/ml).
The patients come from Pediatric Department of San Matteo Hospital - Pavia, by courtesy of Prof.ssa Patrizia Comoli.

3.2 Standard cytogenetics

Bone marrow aspirate (sternal or iliac crest) of patients with AML was obtained at the time of the diagnosis, cultured and harvest (24 and 48 h cultures in Chang BMC and Chang Marrow culture media - Irvine Scientific, Santa Ana, USA) using standard cytogenetic protocols according to SIGU Guidelines.

Briefly:

- Dispence 10 ml of Chang BMC/Chang Marrow culture media into a tubes and add 0,5ml of bone marrow sample. Inoculate al 37°C for 24 – 48 hours;
- Inject the cultures with 50 ml of Colcemid (10 ml/ml);
- Recover the cells by low-speed centrifugation (2000rpm, 5 minutes) and stuck off the supernatant to around 3mm above the cell pellet;
- Flick the rube to distribute the cell and add the hypotonic solution KCl (0,56%, 0.075M) for 20 minutes;
- Centrifugate at 2000rpm 5 minutes and remove the supernatant;
- Carefully add 5 ml of fresh Fixative (3:1 ratio of methanol:acetic acid) to the cells while vortexing. Then add 5 ml more of fixative without vortexing for a total of 10 ml.
- Centrifuge at 2000rpm for 5 minutes. Remove supernatant and resuspend cells. Add 5 ml of fixative to each tube. Repeat this procedure twice.
- Store the cell at 4 °C
- After gently resuspending the pellet, pipette three drops of the cell suspension from a distance of about 2 cm onto a slide which is tilted at an angle of about 45° and allow the suspension to roll across the slide.
- After thoriugh drying the slides may be stained in a fresh solution of Quinacrina dihydrochloride for at least 15 minutes
- Slides are ready for examination.

For each case a minimum of 20 metaphases were visualized under microscope and analyzed using QFQ-banding at a resolution of 350bands.

The chromosomal aberrations observed were described according to ISCN (Intenational System of Cytogenetic Nomenclature).

3.3 Fluorescent in situ Hybridization, FISH

FISH was performed according to general procedural recommendations by Cytocell OGT.

Briefly, the main steps are:

- dehydrate the slide in an ethanol series (70%-85% and 100%) each for 2 minutes at room temperature
- pre-denaturation of the sample slide and probe at 37°C for 5 minutes
- denaturation at 75°C for 2 minutes
- hybridization, overnight at 37°C in a humidity chamber.
- post hybridization washes and air-dry at room temperature
- drain the slide and counterstaining with DAPI/Antifade onto each sample before viewing with a fluorescent microscope.

Temperature and buffer concentration (stringency) of hybridization/washing are important: lower stringency can result in a non-specific binding of the probe to other sequences, and higher stringency can instead result in a lack of signal, as well as an incomplete denaturation step.

3.4 Comparative Genomic Hybridization Array, CGH- ARRAY

Genomic DNA was isolated and purified from mononuclear/blasts cells by using Ficoll-paque gradient centrifugation (Biochrom GmbH Leonorenstr 2-6 – Berlin, Germany) and from bone marrow-derived fibroblasts.

DNA was obtained with DNA mini Kit Quiagen extraction Kit, following the manufacters protocol (QIAGEN, Hilden, Germany www.qiagen.com).

Sex-matched normal human DNA (Kreatech – Amsterdam, Holland) was used as reference.

The OD 260/280 method on a photometer (Nanophotometer IMPLEIN, Munchen Germany) was employed to determine the appropriate DNA concentration and its high quality.

Array-CGH analysis was performed by using Oxford Gene Technology's (OGT) CytoSure Hematological Cancer + SNP 8x60k array platform, containing about 60 000 probes (1 probe every 68Kb in high density gene regions, 1 probe every 117Kb in the backbone; LOH resolution is 30Mb) and OGT CytoSure ISCA v2 4x180k array platform, containing about 180 000 probes (1 probe every 19Kb in high density gene regions, 1 probe every 25Kb in the backbone).

The experiment was performed according to the Cytosure OGT protocols.

Briefly:
Genomic DNA (1 μg) was mixed with 10 μl of Random primer and Reaction buffer solutions and MilliQ water to a total volume of 18 μl. The mix was denaturated at 99° C for 20 minutes and then incubated in ice for 5 minutes.

Each sample was added with 10 μl of nucleotide mix, 1 μl of Cy3-dCTP (test sample) or 1 μl of Cy5-dCTP (reference sample) and with 1μl of Exo-Klenow. The samples were incubated at 37°C for at least 2 hours and then subsequentely purified using Amicon Ultra 30K purification kit (Millipore).

Test and reference DNA were pooled and mixed with 5 μg of Human Cot I DNA (1mg/ml), 11 μl of Blocking buffer (Agilent Technologies) and 55 μl of Hybridization buffer (Agilent Technologies). Before hybridization to the array.

After samples' denaturation at 94°C for 3 minutes and 37°C for 30 minutes, probes were applied to the slide using an Agilent microarray hybridization station. Hybridization was carried out for 24/40 hrs at 65°C in a rotating oven (20 rpm). The array was disassembled and washed according to the manufacturer protocol with wash buffers supplied by Agilent.

The slides were dried and scanned at 2mm using Innoscan 710 Microarray scanner (Carbone, France).

The software automatically determines the fluorescence intensities of the spots for both fluorochromes performing background subtraction and data normalization, and compiles the data into a spreadsheet that links the fluorescent signal of every oligo on the array to the oligo name, its position on the array and its position in the genome.

The genomic locations were based on Genome Reference Consortium GRCh37.

Aberration segments were reviewed using GRCh37 hg19 of UCSC Genome Browser (http://genome.ucsc.edu/index.html).

We annotated all detected copy number variations (CNVs) and CNV-encompassed genes across public databases: Genomic Variants Database (DGV) (http://dgv.tcag.ca/dgv/app/home), DECIPHER (https://decipher.sanger.ac.uk/), Clinical Genome Resource (ClinGen) and ISCA (http://www.clinicalgenome.org), OMIM (http://www.omim.org), PubMed (http://www.ncbi.nlm.nih.gov/pubmed).

3.5 Polymerase Chain Reaction (PCR) and Reverse-Transcription Polymerase Chain Reaction (RTPCR)

RNA was isolated with RNeasy Mini Kit QIAGEN, according to manufactures' recommendations (www.QIAGEN.com).

cDNA was obtained with "Superscript VILO cDNA synthesis kit" by Invitrogen (Thermofisher), according to manufacturer’s recommendations (www.thermofisher.com)

Translocation and inversion are performed following the "Experteam Translocation KIT" manufacturer’s recommendations (www.experteam.it).

NPM1 and FLT3 analysis were performed according to literature (Falini B et al., 2005 New England Journal of Medicine; Nakao M et al., 1996 Leukemia vol.10)

3.6 Sanger sequencing

DNA sequencing is a post-PCR analysis, that permit to identify and/or confirm mutations.

All the sequencing reaction in our laboratory are performed by using Big Dye Terminator v1.1 (Applied Biosystem _ Forster City CA) and run by Abi Prism 310 genetic Analyzer (Applied Biosystem _ Forster City CA).

To detect raw data we commonly use Sequencing analysis v5.6 software (Applied Biosystem _ Forster City CA).

3.7 Bone marrow-derived Fibroblast cells culture

Bone marrow was collected and anticoagulated with heparin.

Briefly, the cell were washed with PBS and then were resuspended in low glucose DMEM medium containing 10% fetal bovine serum and 1% penicillin-streptomycin.

The cell was inoculated into cell culture flask with 1x106 cell/mL density and cultured (37oC, 5%CO2).

After 4-6 days, the culture medium was replaced to remove the non-adherent cells, the ramaining aderent cells are mostly fibroblasts.

When the cellular confluence rate 80%, the cell were collected for DNA extraction to performe aCGH.

4 – RESULTS

4.1 AML PATIENTS

From a series of hematologic malignancies sent for diagnostic purpose to the laboratory of Citogenetica e Genetica Medica (Ospedale di Circolo e Fondazione Macchi, Varese) in the period 2010-2016, we selected 26 AML samples on the basis of the availability of hight quality DNA or fixed cells suitable for a-CGH / SNP-CGH procedure.

Diagnosis of primary AML was estabilished at the Hematology Department of our hospital by conventional morphology, histologic, cytochemical, immunophenotypic and genetic criteria, following the WHO guide.

We divided cases into 2 groups based on conventional karyotypes and, sometimes, FISH:
group A consisted of 22 patients with normal karyotype; group B consisted of four patients with complex karyotype.

Karyotype was reported based on the analysis of at least 20 metaphases.
Cytogenetic nomenclature followed ISCN criteria enforced at time of diagnosis.

An informed written consent was obtained during the onset of the disease of all patients; clinical information, such as age, sex, cytogenetic/molecular markers, history and survival were retrospectively obtained.

4.1.1 Group 1 - AML with normal karyotype

QFQ-banding karyotype analysis of non-stimulated bone marrow samples (24-48h), at the onset of AML disease, was normal in all 22 patients analyzed (46, XX or 46, XY) in a mimimun of 20 observed metaphases.

To assess the possible existence of unbalanced chromosomal abnormalities and/or cryptic rearrangements, we performed in all AML patients CGH array analysis + SNP with 8X60K Hematological cancer SNP (Cytosure OGT) and the following molecular tests, when requested by haematologists during the onset of AML: t(15;17), t(8;21), inv(16), NPM1, FLT3, IDH1/IDH2.

Copy number changes frequently present in healthy population and well-know as common polymorphisms are not reported in this study.

Table 5 describes the genomic imbalances (CNV Gain and Loss and LOH) and the results of molecular analysis in tested patients.
Empty cells indicate that the molecular test was not required.

11 out of 22 patients showed a normal genomic setting, without sub-microscopic aberrations (Patient number 3, 5, 7, 11, 12, 13, 14, 15, 16, 18 and 20).
Among them, seven cases (Patient number 3, 12, 13, 14, 15, 16 and 18) showed only molecular genic mutation: NMP1 was found mutated in patients 15, 16 and 18; FLT3 in patients 13 and 14.
Patient 3 and 12 were found mutated in both NPM1 and FLT3 genes.
Four cases (Patient number: 5, 7, 11 and 20) resulted normal also for molecular tests requested.
Two cases (Patient number 1 and 21) showed CNVs and Genic Variation; two cases (Patient 9 and 19) showed only LOH; three cases (Patients number 4, 10 and 17) only CNVs.
Three cases (Patient number 6, 8, 22) showed both CNVs and LOH and one case (Patient 2) showed CNVs, LOH and genic mutation (Table 6).

PATIENT	SEX and AGE	COPY NUMBER VARIATION, CNV						LOSS OF HETEROZIGOSITY		GENIC MUTATION					OUTCOME
		GAIN			LOSS										
		LOCATION	SIZE	POLIMORPHISM	LOCATION	SIZE	POLIMORPHISM	LOCATION	SIZE	t(15;17)	t(8;21)	inv(16)	NPM1	FLT3	
1	M, 69	3q26.32q26.33	404 Kb		//	//	//	//	//				M *	n	CR, RE, PR, CR, RE, in treatment
2	F, 73	//	//	//	7q22.1	40Kb	Partial	Chr X	106 Mb		n	n	M *	n	R, RE, D
3	F, 71	//	//	//	//	//	//	//	//		n	n	M *	M *	R in treatment
4	F, 70	Chr 10	132 Mb	NO	//	//	//	//	//		n	n	n	n	CR in FU
5	M, 70	//	//	//	//	//	//	//	//			n	n	n	PR, D
6	M, 70	Chr 4	186 Mb	NO	//	//	//	11q13.2q25	66 Mb		n	n	n	n	R, D
7	M, 42	//	//	//	//	//	//	//	//	n	n	n	n	n	PR, RE, D
8	M, 43	Chr 13	96Mb	NO	17q21.31	632 Kb	Partial	11q13.2q25	69Mb		n		n	n	CR in FU
9	F, 56	//	//	//	//	//	//	2p25.3p11.2	83 Mb				n	n	PR, RE, D
10	M, 77	10q25.1	2,4 Mb	Partial	//	//	//	//	//	n					R, D
11	M, 59	//	//	//	//	//	//	//	//		n	n	n	n	CR
12	M, 53	//	//	//	//	//	//	//	//		n	n	M *	M *	CR, RE, D
13	M, 67	//	//	//	//	//	//	//	//		n	n	n	M *	CR in FU
14	F, 48	//	//	//	//	//	//	//	//		n	n	n	M *	R, BMT, D

PATIENT	SEX, AGE	COPY NUMBER VARIATION, CNV						LOSS OF HETEROZIGOSITY		GENIC MUTATION					OUTCOME
		GAIN			LOSS										
		LOCATION	SIZE	POLIMORPHISM	LOCATION	SIZE	POLIMORPHISM	LOCATION	SIZE	t(15;17)	t(8;21)	inv(16)	NPM1	FLT3	
15	M, 52	//	//	//	//	//	//	//	//		n	n	M*	n	R. in FU
16	F, 74	//	//	//	//	//	//	//	//		n	n	M*	n	CR, RE, D
17	M, 53	4p16.3 7q22.1 17q21.31	53Kb 82 Kb 21Kb	Partial NO NO	-Y	56 Mb	NO	//	//				n	n	CR in FU(High Risk)
18	M, 41	//	//	//	//	//	//	//	//		n	n	M*	n	CR, late RE, BMT, CR in FU
19	M, 78	//	//	//	//	//	//	4q21.3q35.2	102 Kb		n	n	n	n	R in treatment
20	F, 71	//	//	//	//	//	//	//			n	n	n	n	R
21	M, 75	Chr 1q Chr 8	107 Mb 143 Mb	NO	//	//	//	//	//				M*	n	R in treatment
22	F, 75	Chr 16p	32kb	NO	4q32.1q34.3 Chr 5q Chr 7 12p13.2p12.1 Chr 16q	17Mb 80Mb 253 Mb 57Mb 11Mb 44Mb	NO	//	//		n	n	n	n	R, in treatment

Table 5 - Microarray abnormalities detected by Cytosure OGT 8x60K Hematological cancer CGH + SNP and results of molecular tests.
Abbreviations: M (male), F (female), % Polymorphism (% POL), Mutated (M*), Normal (n), Complete Remission (CR), Partial Remission (PR), Relapsed (RE), Refractory (R), Deceased (D), Follow Up (FU)

PATIENTS		SEX and AGE	CLINICAL OUTCOME		
			R	RE	CR
Normal	5	M, 70	X		
	7	M, 42		X	
	11	M, 59			X
	20	F, 71	R		
Genes mutations	3	F, 71	X		
	12	M, 53		X	
	13	M, 67			X
	14	F, 48	X		
	15	M, 52	X		
	16	F, 74		X	
	18	M, 41		X	

Table 6 - Summary of Patients without sub-microscopic aberration and Clinical outcome .
Abbreviations: M (male), F (female), Relapsed (RE), Refractory (R) Complete Remission (CR)

PATIENT	RESULTS				CLINICAL OUTCOME
	aneuploidy/entire arm imbalances	CNVS	LOH	genic mutation	
1		gain 3q26.32q26.33		NPM1	CR, RE, PR, CR, RE
2		loss 7q22.1		NPM1	R, RE, D
4	+10				CR
6	+4		11q13.2q25		R, D
8	+13		11q13.2q25		CR
9			2p25.3p11.2		PR, RE, D
10		10q25.1			R, D
17	-Y	gain 4p16.3 gain7q22.1 gain17q21.31			CR
19			4q21.3q35.2		R
21	+8 +1q			NPM1	R
22	-7 loss5q +16p -16q	loss4q32.1q34.3 loss12p13.2p12.1			R

Table 7- Summary of Patients with entire/total aneuploidy/monosomy, genic mutation and Clinical outcome .
Abbreviations: Relapsed (RE), Refractory (R) Partial Remission (PR) Complete Remission (CR) Deceased (D)

Patient number 1 showed a Gain of copy number on chromosome 3q26.32q26.33 of 404Kb in size (ISCN: arr[hg19] 3q26.32q26.33(178,853,968-179,258,128)x3) (Figure 3).

The 13 genes included in the non-polymorphic duplication cover the 61% of all its extension.

Among them, the OMIM gene PIK3CA (PHOSPHATIDYLINOSITOL 3-KINASE, CATALYTIC, 110-KD, ALPH, OMIM number#171834) is interrupted at its start point.

the OMIM genes KCNMB3 (#605222), MNF1 (#608506), GNB4 (#610863) and the gene RP11-360P21.2; SNORA25; LRRFIP1P1; ZNF639; RP11-255C15.3; RP11-145M9.4; AC007620.3; RP11-145M9.2; MTHFD2P7 are completely duplicated but non-correlated with onco-haematological disorders.

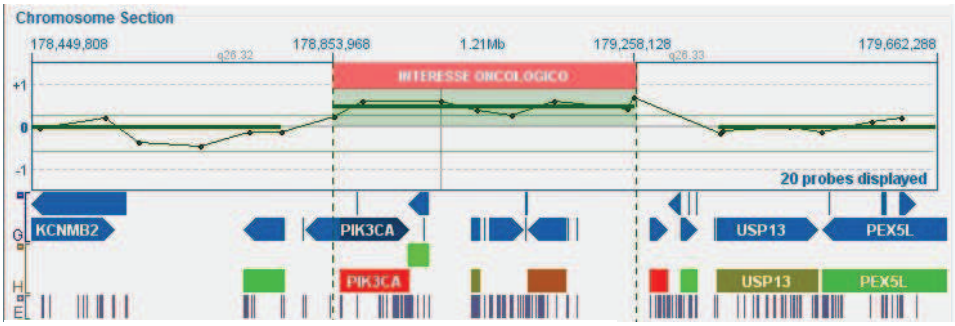
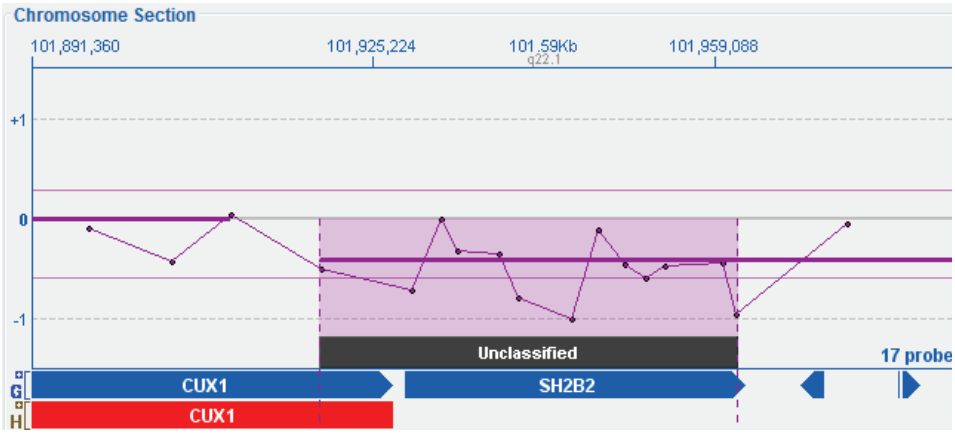


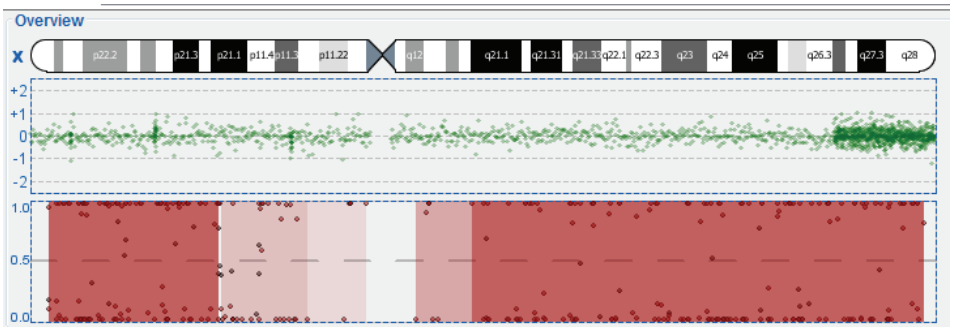
Figure 3 - Gain of 404 Kb on chromosome 3q26.32q26.33.

Patient number 2 showed a 90% mosaic deletion on 7q22.1 of 41Kb in size, that encompass the interrupted CUX1 gene (CUT-LIKE 1, OMIM number#116896) and the entire SH2B2 gene (SH2B ADAPTOR PROTEIN 2, OMIM number#605300) (ISCN: arr[hg19] 7q22.1(101,919,899-101,961,043)x1)

Moreover, patient 2 showed also a significative LOH on entire chromosome X, both p and q arms of 29 Mb (Xp22.33p21.1 start: 3,173,509; end: 32,400,206) and 77 Mb (Xq13.3q28 start: 75,876,163; end: 153,390,510) in size respectively.



A



B

Figure 4 – Patient 2 mosaic deletion on chromosome 7q22.1 (Panel A) and LOH on chromosome X (Panel B)

Patient number 4, represented in figure 5, showed a gain in 10p15.3p11.1 and a gain in 10q11.21q26.3, resulting in trisomy of entire chromosome 10. (ISCN: arr[hg19]10p15.3p11.1(139,069-39,075,180)x3; arr[hg19] 10q11.21q26.3(42,620,814-135,405,947)x3).

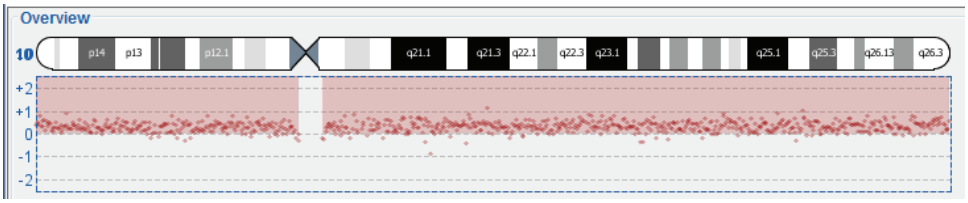


Figure 5 - Trisomy of chromosome 10

Patients number 6 showed a gain of Chromosome 4: arr[hg19] 4p16.3p11(106,265-49,616,789)x3; 4q12q35.2(52,718,771-190,925,290)x3 and a 66 Mb on Chromosome 11q LOH, as shown in figure 6.

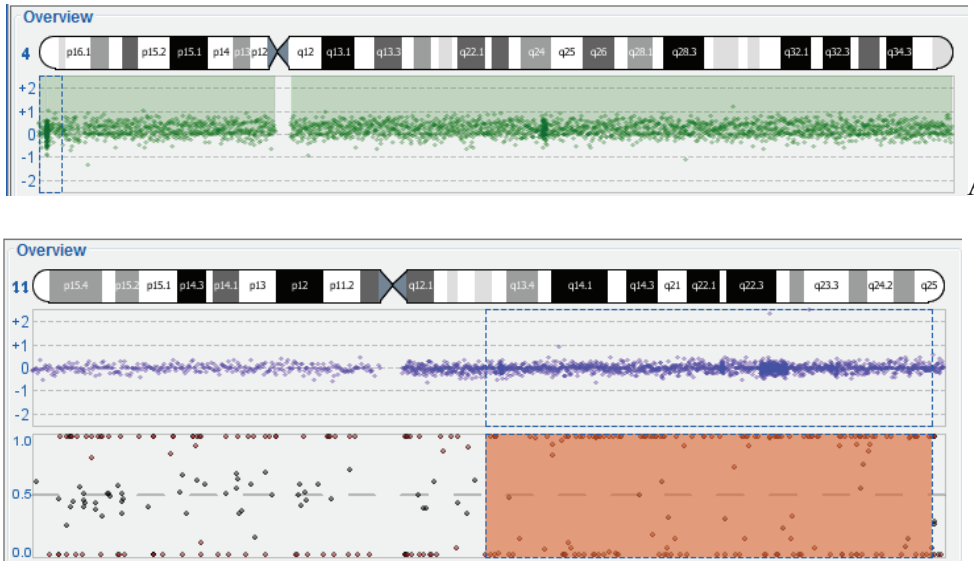


Figure 6 – Trisomy of chromosome 4 (Panel A); LOH of 66 Mb in chromosome 11q13.2q25 (start: 67,169,569; end: 133,167,706)(Panel B)

Loss of heterozigosity encompasses 1412 gene, with a gene coverage of 57%.
Among them, 90 genes are OMIM correlated to multiple diseases.

Patient number 8 showed a LOH on chromosome 11q13.1q25 (start:65,485,307; end: 134,586,308), a trisomy of chromosome 13, and a loss of 632 Kb on chromosome 17: arr[hg19] 17q21.31(43,710,381-44,342,442)x1. (Figure 7, panel A, B, C).

Loss of heterozigosity encompasses 1412 gene, with a gene coverage of 57%; 90 genes are OMIM correlated to multiple diseases.

CNV on chromosome 17, includes 17 genes non correlated with haematological disorders:
CRHR1-IT1; CRHR1; RP11-105N13.4; RP11-293E1.2; RP11-293E1.1; MAPT-AS1; SPPL2C; MAPT; RP11-669E14.4; STH; KANSL1; RP11-669E14.6; RNU7-101P; KANSL1-AS1; Y_RNA; RP11-259G18.2; RP11-259G18.3.
Although the gene coverage is more than 90%, the deletion is completely polymorphic.

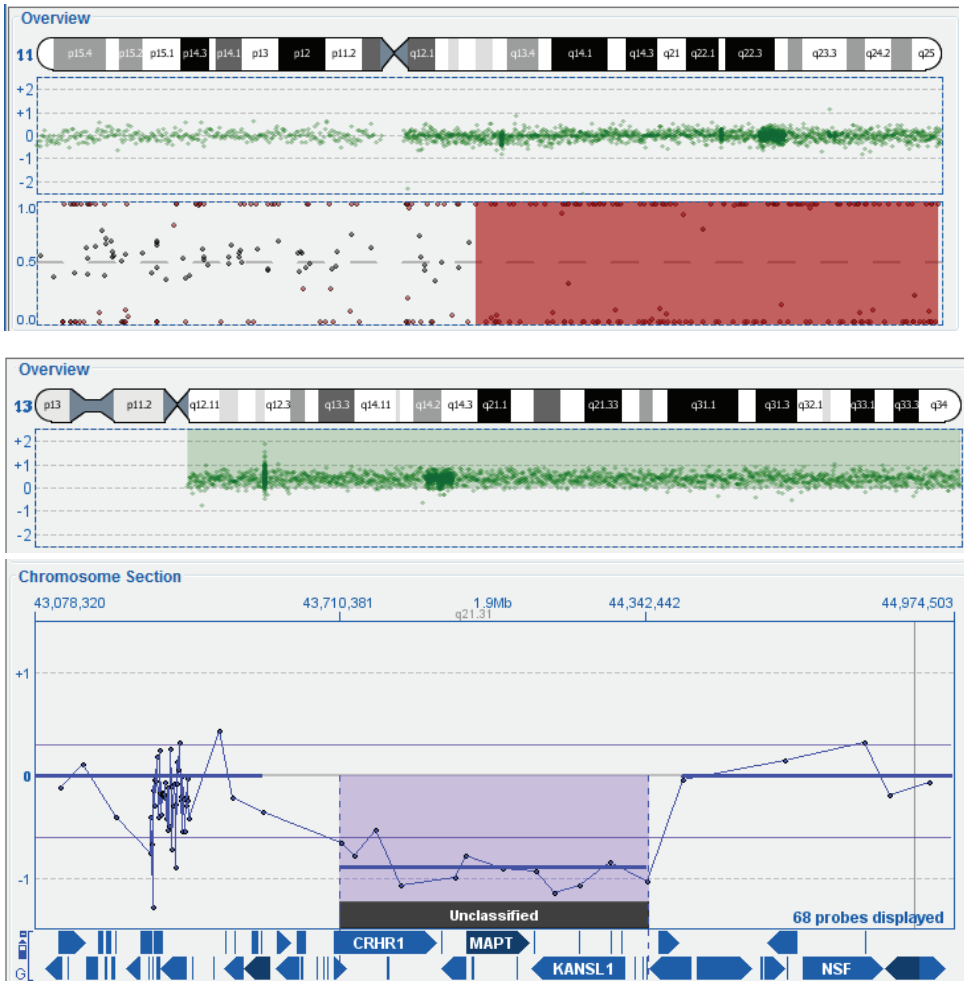


Figure 7 – LOH on chromosome 11q (Panel A), Gain of chromosome 13 (Panel B); Deletion of 632 Kb encompasses the MAPT gene on chromosome 17 (Panel C)

Patient number 9 showed a LOH of 84 Mb, which encompasses the quite entire short arm of chromosome 2 (start:852,240; end: 84,177,129) (Figure 8)

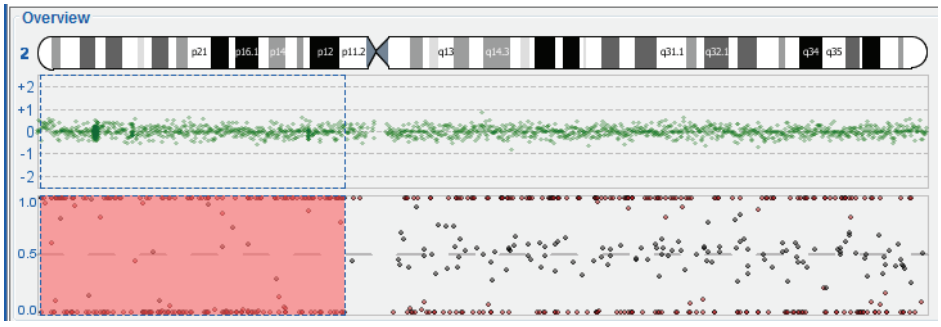


Figure 8 – LOH on chromosome 2

Patient number 10 showed a non-polymorphic duplication of 2,4 Mb in size on chromosome 10q25.1: arr [hg19]10q25.1(108,483,415-110,887,166)x3 (figure 9).

This region encompasses 9 genes: SORCS1; RNA5SP325; RNA5SP326; RP11-215N21.1; PTGES3P5; RP11-163F15.1;RP11-655H13.2; MAPKAPK5P1; RN7SKP278.
SORCS1, interrupted at start point, is the only OMIM gene.

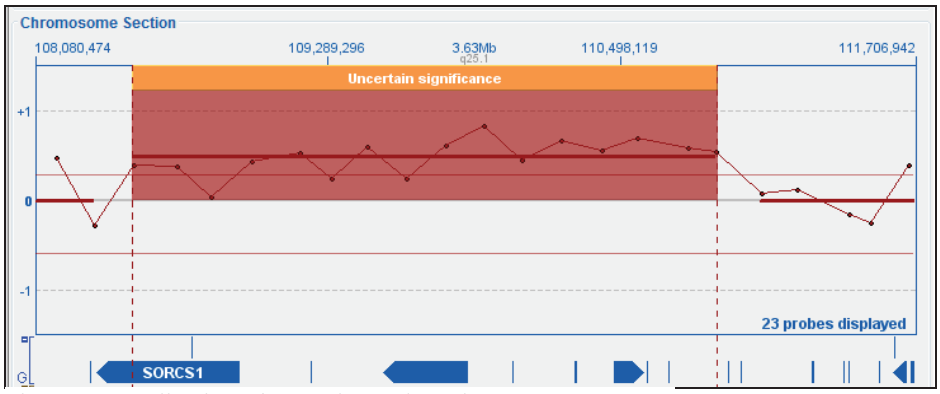


Figure 9 - Duplication of genes located on chromosome 10q25.1

Patient number 17 showed a not polymorphic duplication of 53Kb length on chromosome 4p16.3: arr[Hg19] 4p16.3(1,757,674-1,809,838)x3.
This region includes the FGFR3 gene (FIBROBLAST GROWTH FACTOR RECEPTOR 3, OMIM number#134934) related to OMIM Morbib #100800. (Figure 10, Panel A)
A Copy number gain of 82 Kb on chromosome 7q22.1 interrupt CUX1 gene (CUT-LIKE 1, OMIM number#116896), and duplicate the entire SH2B2 gene (SH2B ADAPTOR PROTEIN 2, OMIM number#605300) (arr[Hg19] 7q22.1(101,879,884-101,961,043)x3). This regions is about 30% polymorphic. (Figure 10, Panel B).
Patient 17 also presented a gain in 17q21.21 encompassing 5 genes: FMNL, SPATA32 and MAP3K14-AS1 are duplicated and interrupted, CTD-2020K17.3; CTD-2020K17.4 are instead completely duplicated: arr [hg19] 17q21.31(43,313,986-43,334,920)x3 (Figure 10, panel C).
A deletion of entire Chromosome Y was also detected by microarrays analysis: arr[hg19] Yp11.32p11.2(132,137-10,012,148)x0 (Figure 10, Panel D).

Figure 10 – Gain on 4p (Panel A), Gain 7q (Panel B), gain 17q (Panel C), Loss of Y chromosome (Panel D).

Patient number 19 showed, as only genomic aberration, the 102 Mb LOH on chromosome 4q21.3q35.2. (start: 87,719,432 end:190,318,109). This region comprise 1242 gene.

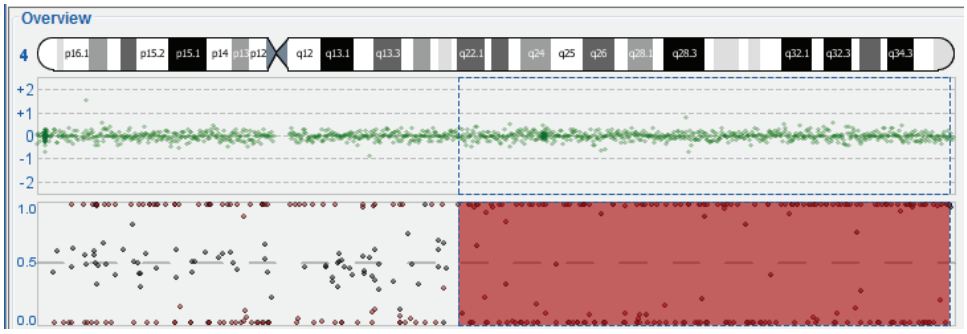


Figure 11 - LOH of 102Kb on chromosome 4

Patient number 21 showed a trisomy of chromosome 8 (arr[hg19] 8p23.3p11.1(123,735-43,803,539)x3; arr[hg19] 8q11.1q24.3(46,942,962-146,147,478)x3) and a 80% mosaicism gain on chromosome 1q21.1q44 of 107Mb in size (arr[hg19]1q21.1q44(142,706,628-249,228,445)x3). this duplication includes 2547 genes (gene coverage 54%) (Figure 12, Panel A and B)

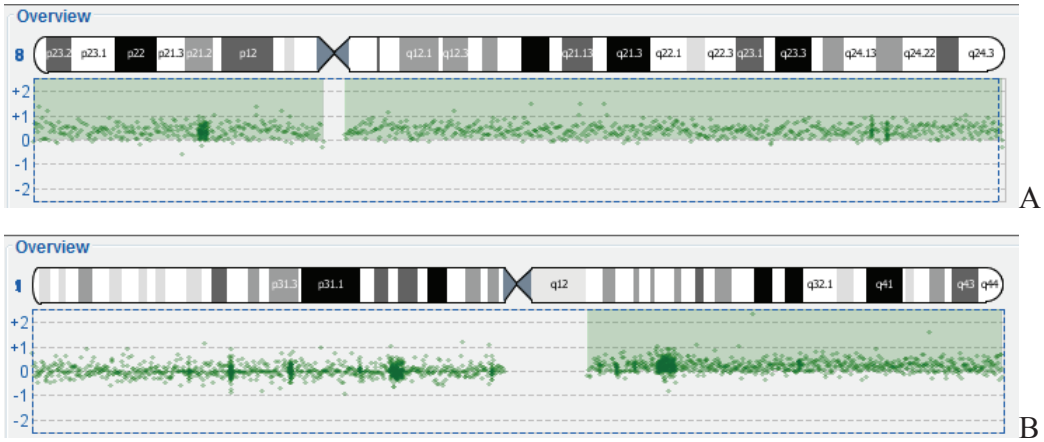


Figure 12 - Trisomy 8 (Panel A) and Loss 1q (Panel B)

Patient number 22 represented in figure 13 showed multiple complex genomic changes in chromosomes 5, 7, 12, 16 and 22 and demonstrated that abnormalities were indeed present.

In Chromosome 4 a deletion of 17.7Mb in size (arr[hg19] 4q32.31q34.3(160,224,719-177,928,614) was detected. 5/183 genes deleted (GK3P; TLL1; PALLD; NEK1; HPGD) are related to OMIM disease (Figure 13, Panel A)

Chromosome 5 presented a deletion of 1245 gene of 80 Mb in size (arr[hg19] 5q14.3q34(86,951,953-167,597,145), resulting in loss of more than one half q arm. (Figure 13, Panel B)

Chromosome 7 is completely deleted: arr[hg19] 7p22.3q36.3 (579,941-159,124,478)x1. (Figure 13, Panel C)

Chromosome 12 showed a loss of 11.74 Mb in size located on 12p13.2p12.1: arr[hg19] 12p13.2p12.1(10,929,829-22,672,718)x1. (Figure 13, Panel D)

Chromosome 16 showed a gain of 32.37Mb on p-arm: arr[hg19] 16p13.3p11.2 (99,414-32,470,683)x3; and a loss on q-arm: arr[hg19] 16q11.2q24.3 (46,511,147-90,053,103)x1). (Figure 13, Panel E)

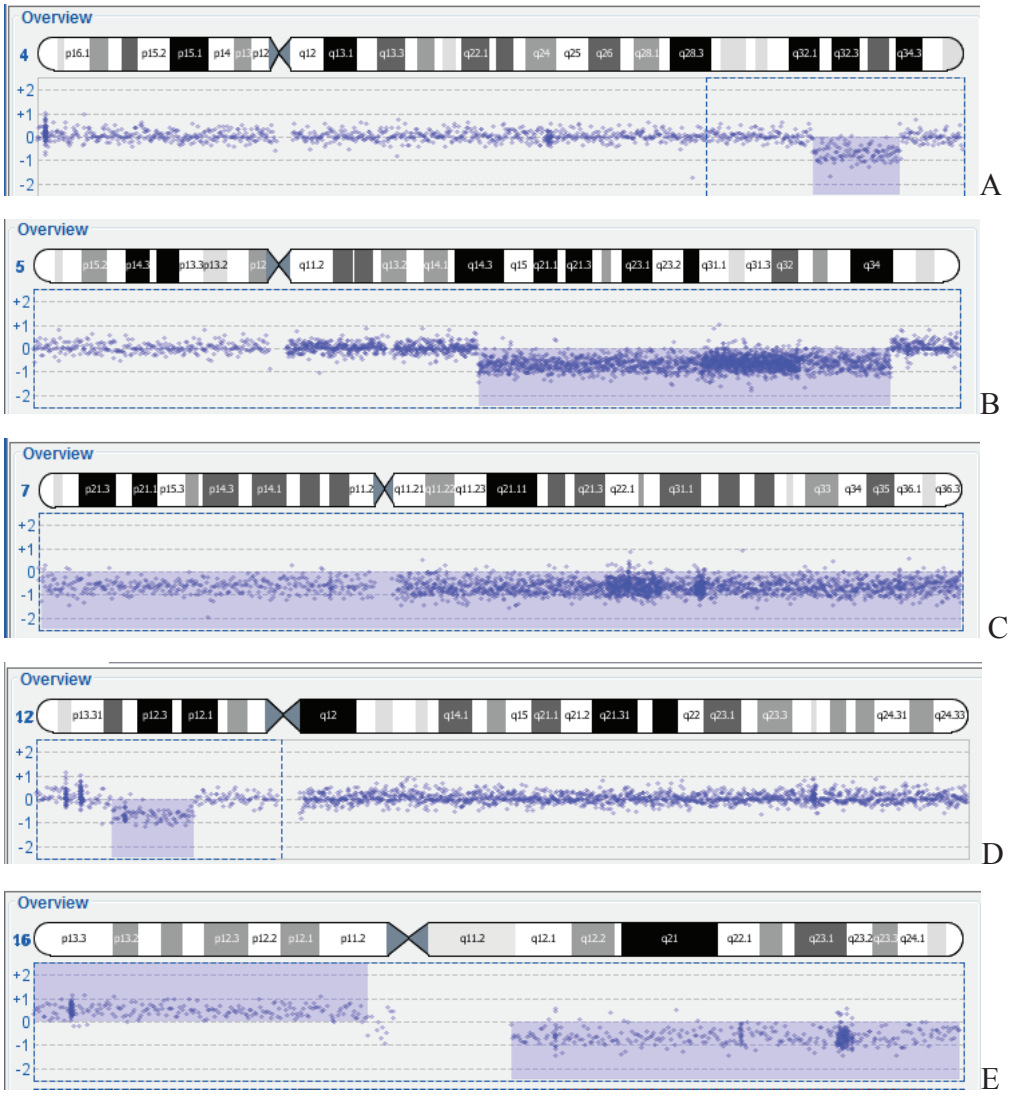


Figure 13 – Loss4q (Panel A), loss5q (Panel B), -7 (Panel C), loss12p (Panel D), Gain16p and loss16q (Panel E)

4.1.2 Group 2) AML subgroup with complex karyotype

Conventional karyotype was performed at diagnosis in 4 patients with not well-defined AML and showed a complex karyotype with multiple rearrangements.

In order to detect cryptic imbalances, CGH array 4x180K Oligo array Platform (Cytosure, OGT) and moleculare tests were performed at the same time.
Table 8 shows the main imbalances and, when present, the corresponding new fusion gene due to translocation.

SEX and AGE	CNV GAIN	SIZE	% POL	CNV LOSS	SIZE	% POL	FUSION GENE	OUTCOME
M, 32	//	//	//	17p11.2-p11.1 17q21.2	1 Mb 1,8Mb	27% 8%	STAT5B-RARA	CR, BMT. In follow up
M,83	//	//	//	2p14-p11.2 11p13 11q12.1-q23.3	15,72Mb 1,123Mb 61,1Mb	0%	?	D
M, 66	7p22.3p15.1 7p14.3p12.3 7p12.1q31.1	23Mb 13,8Mb 49Mb	0%	4q32.3q34.1 5p15.33p15.31 6q24.2q27 7p15.1p14.3 7p12.3p12.1 9p21.3q34.3 12p13.31p12.3 13q11q34 20p12.3p11.23 Xq26.3q28	7,8Mb 7Mb 25,6Mb 4,7Mb 3,5Mb 119,5Mb 7,4 Mb 95,3Mb 12,9Mb 19,3Mb	0%	//	CR, RE, D
M,24	//	//	//	5q14.2q15 5q21.1q32 5q33.1q35.1 5q35.1q35.3 5q35.3 5q35.3	12 Mb 51Mb 23Mb 4Mb 3Mb 3,7Mb	0%	//	n.d.

Table 8 – AML-subtypes patients and the main molecular imbalances

Case 1) APL with STAT5B-RARA fusion gene

A 32-year-old man was admitted to our Hematology Department complaining for fever, cellulitis of the right leg and palpable hepatosplenomegaly.

Examination of peripheral blood indicated a white blood cell count of 6.8 x109/L (neutrophils 0.5 x109/L; lymphocytes 2.7 x109/L; monocytes 0.4 x109/L; blasts 8%), hemoglobin 7.4 g/dL, platelet count 129 x109/L. The bone marrow aspirate showed a 90% blasts infiltration of medium size promyelocytes.

QFQ-banding cytogenetic analysis (Figure 14) on 24 metaphases showed loss of Y chromosome and structural rearrangements of chromosome 17 consisting in a pericentric inversion (p11.2q21) and in a q arm deletion (q21) (24 metaphases) (Figure 15, Panel A); normal male karyotype was found in 5 metaphases.

FISH analysis confirmed the correct position of telomeric probe signals (Figure 15, Panel B) and pericentric inversion of chromosome 17 with RARA probe on 17p arm (Figure 15, Panel C).

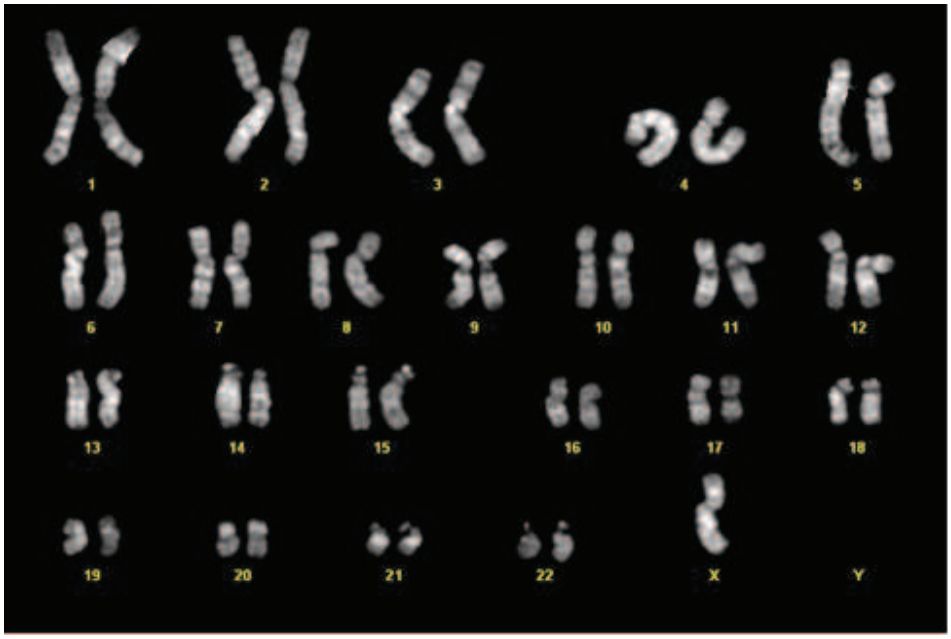


Figure 14 – QFQ-banding karyotype of patient

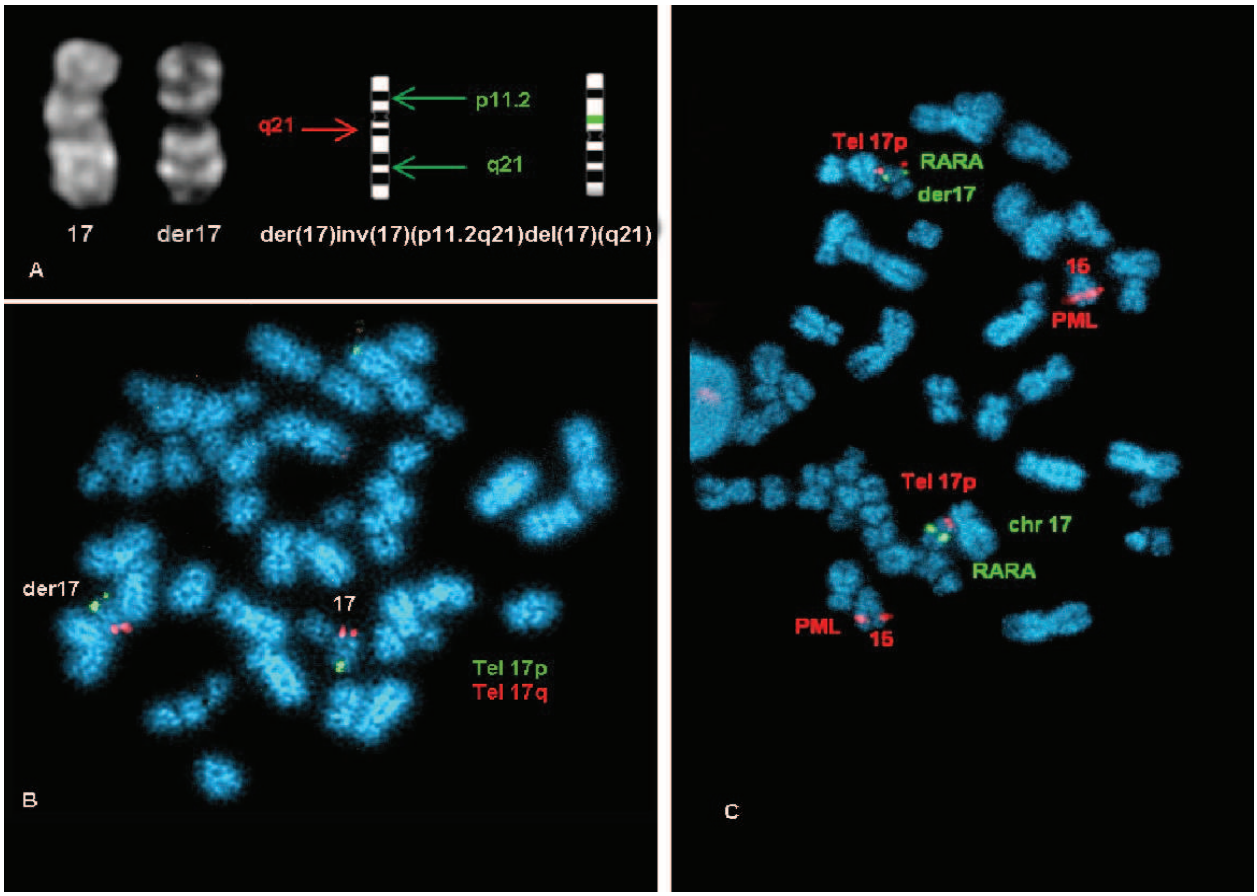


Figure 15 - Chromosomes 17 and der(17)inv(17)(p11.2q21)del(17)(q21) (Panel A); FISH with pretelomeric probes Tel 17p (green, D17S2199), Tel17q (red, D17S2200) (Panel B); FISH with RARA probe (green), PML probe (red) and telomeric 17p probe (red) (Panel C).

We also performed Array-CGH analysis on leukemic cells, in order to better redefine breakpoint region and cryptic imbalances. Two CNVs were detected (Figure 16)

- The first CNV was a 1,01 Mb deletion in 17p11.2p11.1 from start point 21,195,578 to end point 22,205,792. This CNV is polymorphic for 27% of its extension (DGV database) and overlaps 3 OMIM genes: MAP2K3 (OMIM number #602315) interrupted by the start point, KCNJ2 (OMIM number #602323) and KCNJ18 (OMIM number #613236) that is completely deleted.
- The second CNV is a 1,827 Mb deletion in 17q21.2 from 38,526,212 to 40,353,672. This deletion is constitutionally polymorphic for only 8% of its extension, includes 46 full deleted OMIM gene and interrupts STAT5B gene, within the exon ENSE00012935555.

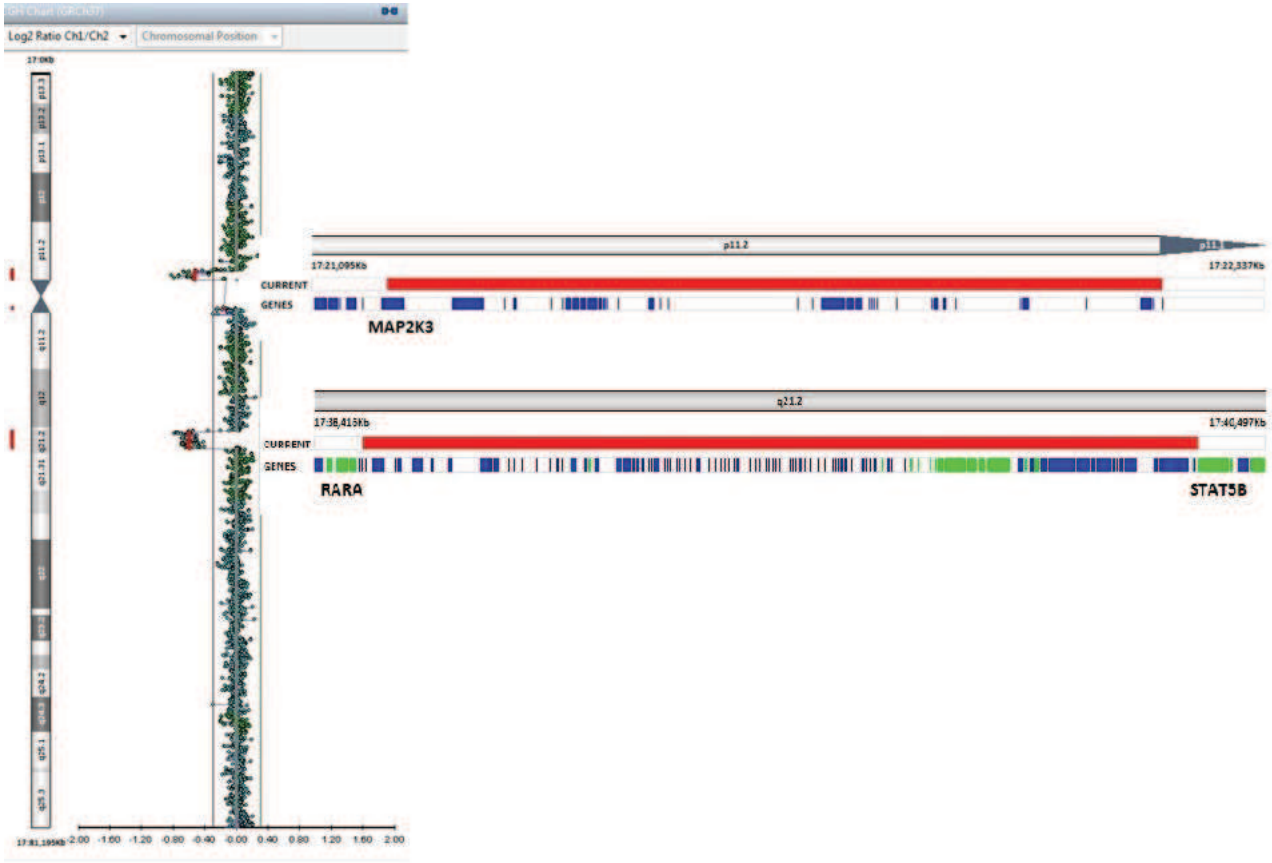


Figure 16 - Array CGH-180K 17p11.2-p11.1 and 17q21.2 deletions.

The final karyotype resulting from cytogenetic, FISH and array-CGH investigations, according to ISCN 2013, was:

45,X,-Y,der(17)inv(17)(p11.2q21)del(17)(q21)[24]/46,XY[5].ish
der(17)(p13)(D17S2199+)(p11.2)(RARA+)(q25)(D17S2200+).
arr[hg19] 17p11.2p11.1(21,195,578-22,205,792)x1,17q21.2(38,526,212-40,353,672)x1

Molecular analysis for PML-RARA [t(15;17)], RUNX1T1-RUNX1 [t(8;21)], CBFB-MYH11 [inv(16)], FLT3-ITD and NPM1 were normal.

Molecular and array-CGH results allowed us to hypothesize the presence of a STAT5B-RARA fusion gene. A specific DNA fragment was amplified with the following primers for STAT5B-RARA:

STAT5B-F1: 5’-GCCGTGCCTGACAAAGT-3’; STAT5B-F2: 5’-ACATCTCAAGCCTCATTGGA-3’; RARA-R1: 5’-TCTTCTTGTTTCGGTCGTT-3’; RARA-R2: 5’-TGTTTCGGTCGTTTCTCAC-3’. The PCR primers were designed using Primer-BLAST.

The nucleotide sequencing analysis of the amplified DNA demonstrated STAT5B-RARA fusion. Sequencing analysis showed the presence of a 390 bp PCR fragment encompassing the fusion between exon 15 of STAT5B with exon 4 of RARA (Figure 17)

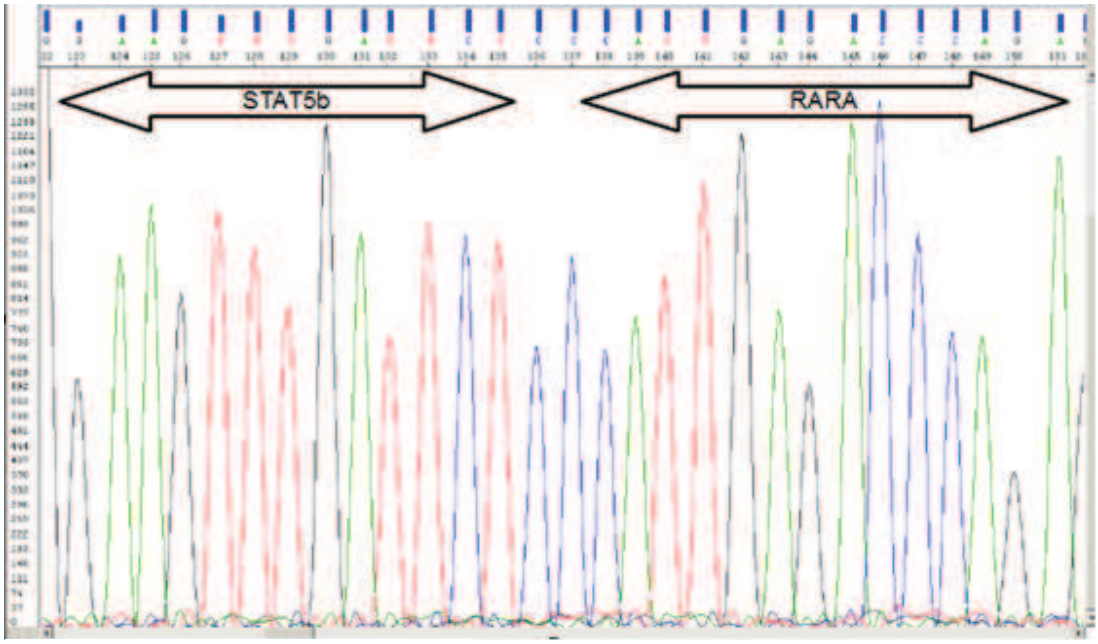


Figure 17 – STAT5b and RARA fusion gene

Case 2) APL with t(4,17)(q12;q21)

A 83 years old man was admitted to our Hematology department for pancytopenia and suspected APL. According to the guidelines, the final karyotype on non-stimulated metaphases was: 46,XY,t(4;17)(q12;q21),del(11)(q12)[20]/46,XY[1].

In order to exclude an unbalanced translocation between chromosomes 4,17 and 11, we performed CGH-array.

Molecular karyotype shows:

- a deletion on 2p14-p11.2 of 15,726Mb in size (arr[hg19] 2p14-p11.2(67,918,036-83,644,034)x1) which encompasses 78 OMIM gene. The maximum overlap between deletion and polymorphic region is 7% of total length.

- a deletion in 11p13 and 11q12.1-q23.3 of 1,123Mb and of 61,1Mb in size respectively (arr[hg19] 11p13832,277,043-33,400,760)x1; arr[hg19] 11q12.1-q23.3(56,482,185-117,566,772)x1). 477 OMIM gene resulted deleted (Figure 18)

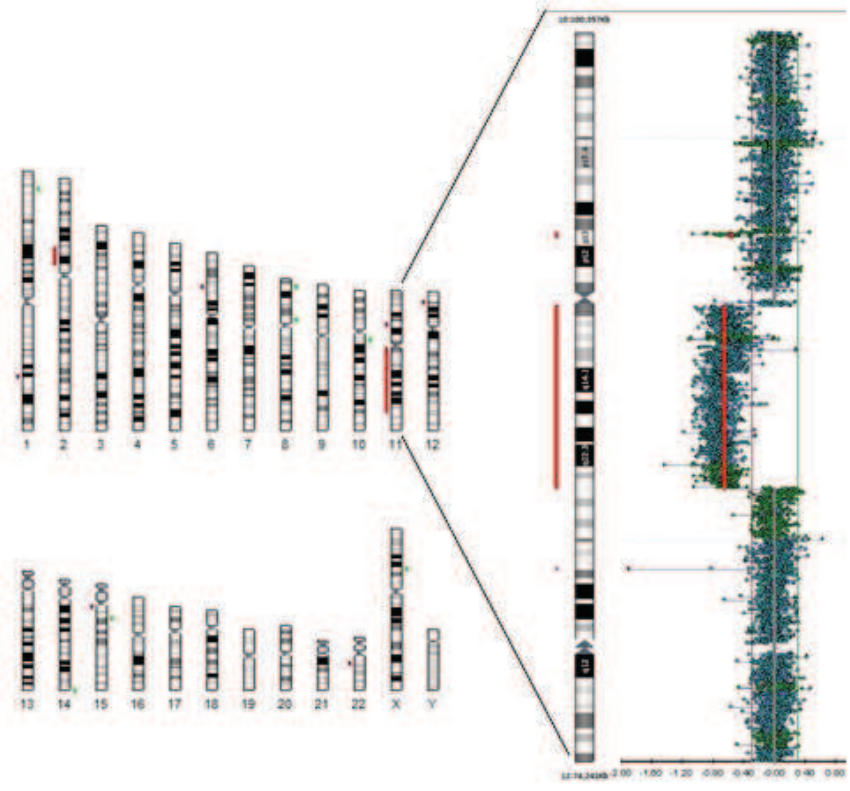


Figure 18 - Molecular Karyotipe (on the left) and enlargement of chromosome 11. (Red line= deletion, Green Line= duplication)

Case 3) Blastic Plasmacytoid Dendritic Cell Neoplasm BPDCN)

Cytogenetic analysis on peripheral blood with more than 30% of circulating blasts showed a monosomy of chromosome 9, typical cytogenetic abnormality of this AML subgroup, and 13 monosomy.
According to ISCN 2013, the final karyotype of 20 non-stimulated metaphases was:
45XY, del(12)(p13), -13, del(20)(p11).

CGH-array analysis on leukemic cells showed several CNVs, as reported in table 9

Copy Number Variation	Size (Mb)
4q32.3q34.1(165,987,693-173,843,632)x1	7,8
5p15.33p15.31(2,539,901-9,630,103)x1	7
6q24.2q27(145,440,205-171,025,493)x1	25,6
7p22.3p15.1 (41,117-28,367,895)x3	23
7p15.1p14.3(28,390,641-33,098,362)x1	4,7
7p14.3p12.3(33,114,898-47,111,872)x3	13,8
7p12.3p12.1(47,134,426-50,634,897)x1	3,5
7p12.1q31.1(50,653,308-113,071,134)x3	49,2
9p21.3q34.3(21,218,577-141,102,496)x1	119,5
12p13.31p12.3(7,778,791-15,692,368)x1	7,4
13q11q34(19,066,607-115,105,783)x1	95,3
20p12.3p11.23(7,782,442-20,747,605)x1	12,9
Xq26.3q28(135,702,412-155,046,701)x1	19,3

Table 9 - Copy Number Variation and corresponding size, expressed in Mega Bases (Mb)

It is interest that genetic imbalance of this rearrangement showed a chromothripsis involving chromosome 7 (Figure 19).

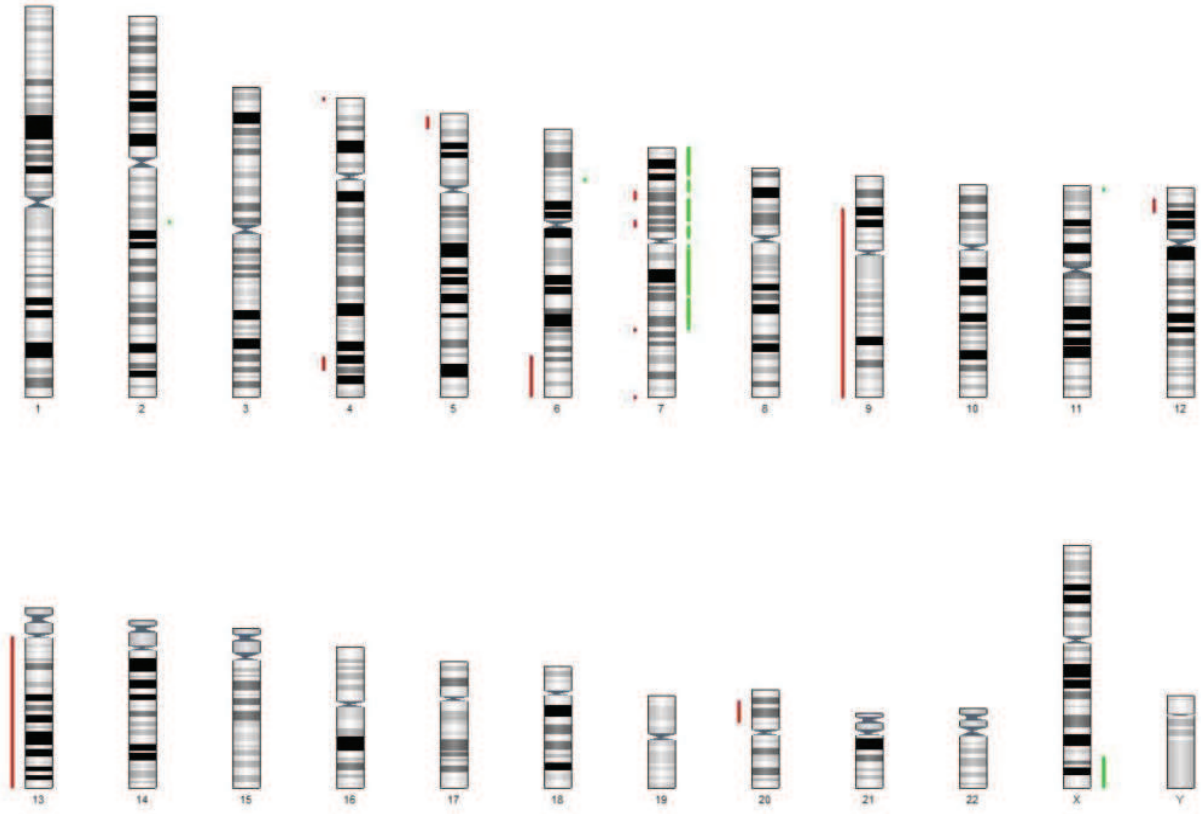


Figure 19 - Molecular karyotype of onset BPDCN. (Red line= deletion, Green Line= duplication)

Case 4) AML with tetraploid karyotype

The first cytogenetic examination revealed in 20/20 analyzed metaphases of the bone marrow cells, a tetraploid range with 92 chromosomes and several genetic abnormalities.

Final Karyotype, according to ISNC 2013 was:

92, XXYY, del(5)(q11.1), del(5)(q21),t(15;17)(q22;q21)x2 [20].

Molecular analysis detected PML-RARA fusion gene due to t(15;17)(q22;q21).

We performed Array CGH in order to better define a deletion on chromosome 5.

Microarrays analysis detected 6 copy number losses on chromosome 5, as reported below in figure 20

The balanced translocation t(15;17) was not detected by this technique.

Copy Number variation	Size (Mb)	Number of GENES deleted
5q14.2q15 (81,936,965-94,037,461)x1	12 Mb	137
5q21.1q32 (98,211,878-149,713,616)x1	51 Mb	870
5q33.1q35.1 (149,824,964-172,654,493)x1	23 Mb	316
5q35.1q35.3 (172,666,340-176,727,833)x1	4 Mb	90
5q35.3 (176,962,031-180,004,080)x1	3 Mb	95
5q35.3 (176,962,031-180,696,832)x1	3,7 Mb	136

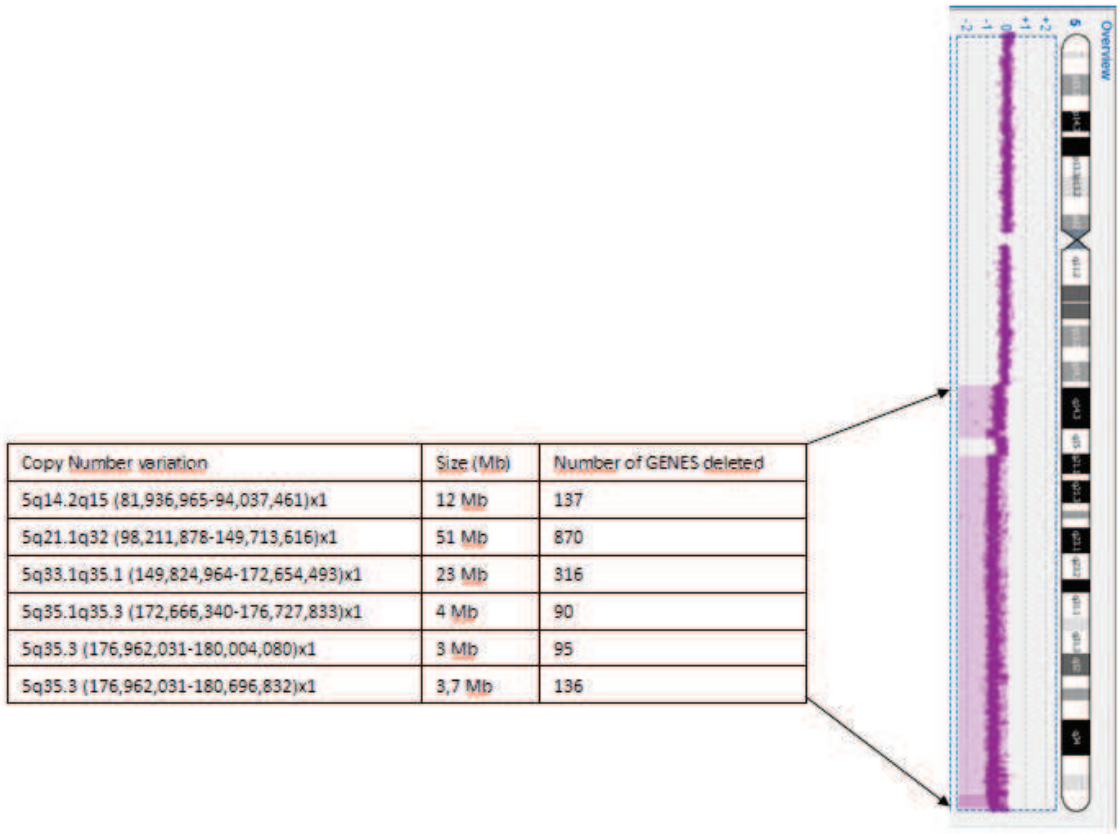


Figure 20 - Chromosome 5 copy number deletions, size expressed in MegaBases and number of genes deleted.

4.2 POST KIDNEY TRANSPLANTED PATIENTS

In order to evaluate constitutional CNVs that might predispose to post-transplantation lymphoproliferative disease, we analyzed twenty-two renal transplated patients at Pedriatric Nephrology Unit IRCCS Giannina Gaslini Institute, Genoa.
Samples from this patients were crio-preserved at Pediatric Hematology/Oncology Department, Fondazione IRCCS Policlinico San Matteo, Pavia of San Matteo Hospital (Pavia).

This study includes a range of pediatric-adolescent patients, male and female, with different age, ranging from 3years old to 18years old, and all EBV negative at time of transplantation (Table 10)

Patients were further grouped into four main categories, based on clinical post-transplantation manifestations (Table 11):

Group 1: patients with PTLD (number: 10, 11, 12, 13, 14, 15, 16, 17);

Group 2: patients without PTLD and EBV- High Viral Load (more than 1000 copies/ml). This group is correlate to high risk to develop the onset of lympoproliferative disease (number: 4, 9, 20, 22);

Group 3: patients without PTLD and EBV Low Viral Load (less than 1000 copies/ml)(number: 5, 7, 19,);

Group 4: patients with absence of clinical manifestations (number: 1, 2, 3, 6, 8, 27, 30).

PATIENT	SEX, AGE	EBV status after transplantation NEG= Negative POS= Positive	REASON OF RENAL TRANSPLANTATION	CLINICAL MANIFESTATION / Viral Load
1	F, 17	NEG	nephronophthisis	//
2	M, 15	NEG	Alport syndrome	//
3	F, 8	NEG	family focal glomerulosclerosis	//
4	F, 15	NEG	hemolytic uremic syndrome	High Viral Load
5	M, 14	POS	renal hypodysplasia	Low Viral Load
6	F, 4	NEG	Congenital nephropaty post FANS	//
7	M, 12	POS	n.d	Low Viral Load
8	M, 18	NEG	Recessive polycystic kidney	//
9	M, 16	POS	n.d	High Viral Load
10	**M, 16**	**n.d**	**n.d**	**PTLD**
11	**M, 5**	**NEG**	**diffuse mesangial sclerosis**	**PTLD**
12	**F, 3**	**NEG**	**renal dysplasia**	**PTLD**
13	**F, 11**	**NEG**	**nephronophthisis**	**PTLD**
14	**M, 11**	**POS**	**Alport syndrome**	**PTLD**
15	**M, n.d**	**NEG**	**n.d**	**PTLD**
16	**M, 4**	**n.d**	**n.d**	**PTLD**
17	**M, 4**	**NEG**	**n.d**	**PTLD**
18	M, 15	NEG	focal glomerulosclerosis	//
19	M, 12	POS	Reflux nephropathy	Low Viral Load
20	M, 9	POS	Lesch-Nyhan Syndrome	High viral load
21	M, 26??	NEG	renal hypodysplasia	//
22	F, 13	POS	Bor syndrome	High Viral Load

Table 10 - Coort of Kidney-transplanted patients

Group	1	2	3	4
Clinical manifestation	PTLD	High Viral Load	Low Viral Load	Control Patients
Patient	10, 11, 12, 13, 14, 15, 16, 17	4, 9, 20, 22	5, 7, 19	1, 2, 3, 6, 8, 18, 21

Table 11 – Schematic rappresentation of groups based on clinical post-transplantation manifestations.

We performed a whole genome analysis by using 4x180K array platform (OGT, Cytosure) and we detected all the CNV listed in the table below.

Chromosomal Band	PATIENTS																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1p21.1		x					x		x	x	x			x		x			x	x		x
1q31.3																	x					
1p34.3															x							
1p36.13														x								
1p36.33	x																					
1q21.1														x								
1q44														x								
1q31.1			x																			
1q31.3							x															
2p22.3																					x	
2p11.2p11.1			x																			
2p11.2					x																	
2q13	x												x	x								
2q37.3	x	x																				
3p24.3	x																					
3q26.1		x		x			x				x				x	x	x		x			
3q29													x									
4p11																		x				
4p16.1																x	x					
4q13.2		x	x	x						x	x	x		x			x	x		x		
4q22.3q23																	x					
4q31.2												x										
5p15.1				x																		x
5p15.3										x								x			x	
5p13.2																					x	
5q13.2			x				x					x						x			x	
5q35.3		x																				
6p21.32											x											
6p22.2							x														x	

Chromosomal Band	PATIENTS																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
6p25.3									x						x					x		x
6q27	x		x																			
7p21.3					x																	
7q22.1					x								x									
7q31.1						x																
7q33																		x				
7q35				x				x		x										x	x	
7q36.3					x	x																
8p23.1	x	x	x		x		x		x	x	x					x		x	x			
8p11.22					x				x						x		x	x			x	
8q21.13						x																
9p13.1		x	x						x	x			x							x		
9p11.2			x							x			x									x
9p21.3																			x			
9p22.2					x																	
9p23											x											
9p24.3				x		x												x				
9q12										x										x	x	
9q13										x										x	x	
10q21.1																			x			
10q23.1			x				x															
11p14.3	x																					
11p11.12			x																			
11q14.1					x																	
12p12.3																					x	
12q12														x								
12q14.2																x						
14q11.2	x			x		x			x			x								x		
14q32.33									x		x	x	x									
15q11.2	x		x	x		x									x					x		

Chromosomal band	PATIENTS																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
15q11.1q11.2		x			x		x										x					x
15q13.2																					x	
15q14			x									x										
15q26.3													x		x	x						
16p11.2p11.1		x																			x	
16p11.2										x				x								x
16p12.2				x																		
16q11.2																				x		
16q24.3					x																	
17q12															x							
17q21.31	x									x			x							x	x	
18q21.1								x														
18q22.3							x															
20p12.3																			x			
20p13				x																		
21q22.11																x						
22q11.1		x										x										
22q11.2	x					x											x					x
22q11.23q12.1															x							
Xp11.23																			x		x	
Xp22.33		x			x	x	x		x		x		x	x		x		x				x
Xq22.2	x																					
Xq11.1										x												
Xq26																				x		
Xq28												x										
Yp11.32		x							x							x		x				
Yq11.223		x			x		x	x	x							x	x					
Yq11.23		x			x			x	x							x	x					
Yq11.23q12								x						x								

Table 12 – List of all genomic aberration detected by 180K oligo array platform

The most recurrent regions of genomic imbalance in our patients (CNVs) are summarized in the following chromosomal bands (Table 13)

Chromosomal band	PATIENTS																					
	Control							Low Viral			High Viral				PTLD							
	1	2	3	6	8	18	21	5	7	19	4	9	20	22	10	11	12	13	14	15	16	17
1p21.1		G							L	L		L	G	G	G	G			G		G	
2q13	L																	L	L			
4p16.1																					L	L
4q13.2		L	G			L					L		L		G	G	L		G			G
5p15.1											G			G								
5q13.2			G			G	G		L								G					
6p25.3												L	L	G						G		
7q22.1								L										L				
8p11.22						G	L	G				G								L		G
9p13.1												L			G			L				
14q32.33																G	L	L				
15q11.2	G		G	G							G		G							G		
16p11.2														G	G							
17q21.31							G						G		G							
22q11.2	G			G										G								G
Xp22.33		G		L		G		L	L			L/G		G		L		L	L	G		
Yq11.223		G			G			L														G
Yq11.23		G			G			L														G

Table 13 - Recurrent and significative CNVs in post-transplanted patients (C= Control patients, LV=low viral, HV=High viral, PTLD)

Here follow the most frequent anomalies detected in our cohort of patients.

Patient number 14 presented both gain and loss of 1p21 chromosomal region.

The CN loss is located on 1p21.1(103,517,424-104,284,282) is 776Kb in size and encompasses 15 genes (COL11A1; SOD2P1; RP11-347K2.1; RP11-347K2.2; RP11-153F1.2; RP11-153F1.1;RN7SKP285; RNPC3; AMY2B; ACTG1P4; AMY2A; AMY1A; RP5-1108M17.5; AMY1B;AMYP1)

Among them, the OMIM gene COL11A1 (COLLAGEN, TYPE XI, ALPHA-1; OMIM number#120280) is interrupted between exon 3 and 4. This is a non-polimorphic region.

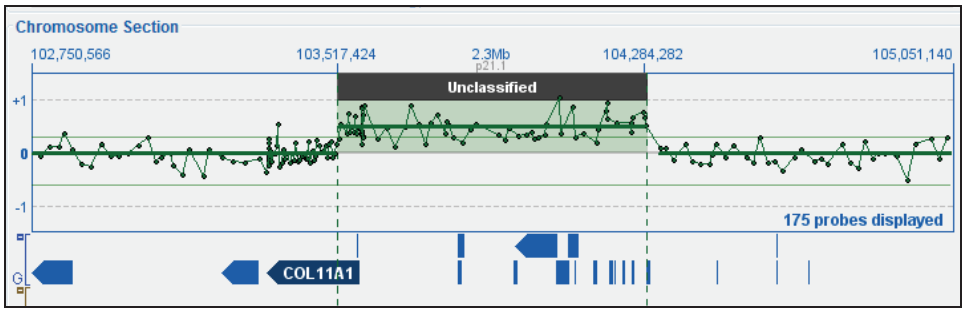


Figure 21- arr[hg19] 1p21.1(103,517,424-104,284,282)x3

Patient number 1 and 13 carried a 496 Kb and 322 Kb deletions respectively, including the start/end point of NPHP1 (NEPHROCYSTIN 1, OMIM numbre#607100) related to Nephronophthisis 1, juvenile (#256100) (figure 22)

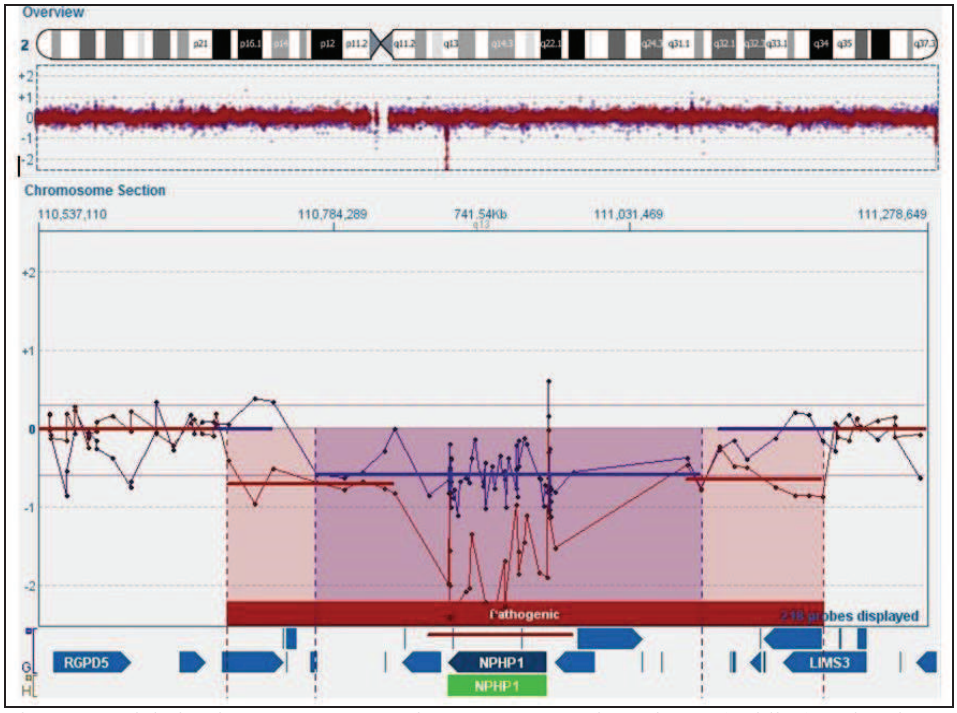


Figure 22 - deletion in 2q13 encompassing NPHP1 gene in patient 3 (red line) and patient 13 (blu line)

Four patients (patients High Viral 9, 20, 22 and patient PTLD 15) presented a genomic aberration involve chromosome 6p25.3.Patients number 9 and 20 reported a copy number variant Loss; patients 15 and 22 reported a copy number variant Gain.

This region is only 40Kb in length and also polymorphic, but DUSP22 gene (dual specificity phosphatase 22) is relevant and correlated with onco-hematological disorders.

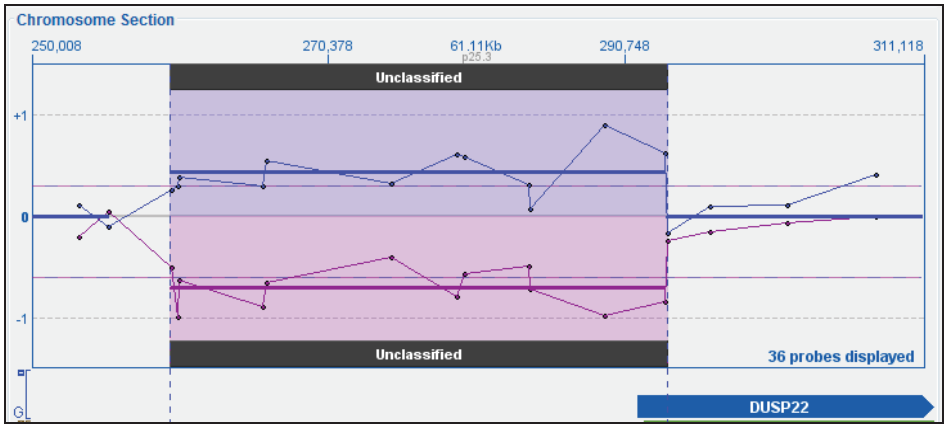


Figure 23 - arr[hg19] 6p25.3(259,531-293,497)x1 (Patient 9 violet line), arr[hg19] 6p25.3(259,531-293,497)x3(Patient 15 blue line)

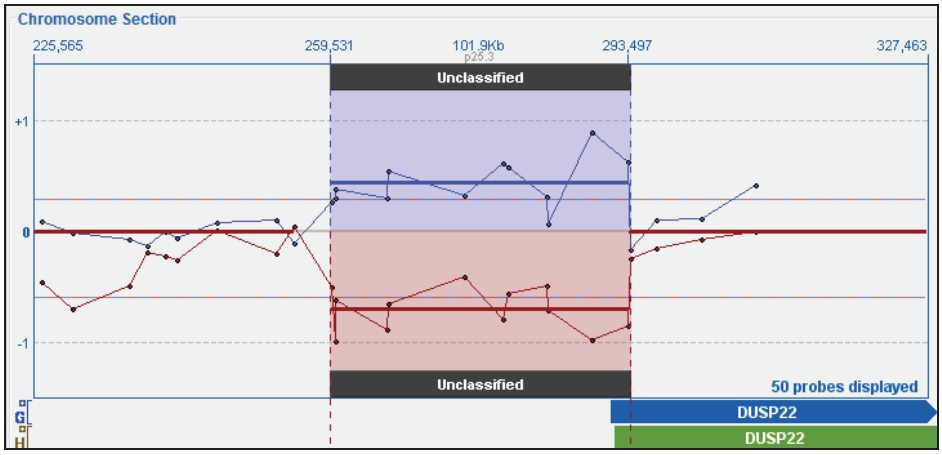


Figure 24 - arr[hg19] 6p25.3(259,531-293,497)x1 (Patient 20 red line), arr[hg19] 6p25.3(259,531-293,497)x3 (Patient 22 blue line)

Both copy number variant duplications and deletions affected chromosome 9p13.1.

Size are very variable, as reported in Table 14.

Patient	ISCN Notation	Size	Number of gene involved
2	arr[hg19] 9p13.1(38,846,938-38,899,396)x3	52,46 Kb	1
3	arr[hg19] 9p13.1(38,791,655-38,951,375)x3 arr[hg19] 9p13.1(40,491,851-40,632,479)x3	159,72 Kb 140,63 Kb	3 16
10	arr[hg19] 9p13.1(38,768,412-38,951,375)x3	182,96 Kb	1
9	arr[hg19] 9p13.1p12(38,789,216-42,010,037)x1	3,22 Mb	63
13	arr[hg19] 9p13.1p11.1(38,822,497-47,312,281)x1	8,49 Mb	208

Table 14 Aberrations in patients 2, 3, 10, 9 and 13 on chromosome 9.

Patients 11, 12,13 reported a CNVs in 14q32.33.

Although this genomic alteration overlap a highly polymorphic region, it is relevant because includes several gene coding for IGHV (Table 15).

Patient	ISCN Notation	Size	Number of gene involved
11	arr[hg19]14q32.33(107,148,712-107,182,881)x3	34,17 Kb	5
12	arr[hg19]14q32.33(106,512,752-106,764,191)x1	251,44 Kb	33
13	arr[hg19]14q32.33 (106,602,112-106,803,289)x1	201,18 Kb	33

Tab 15. Characteristics of duplication and deletions in patients 11 and 13.

Patient number 10 and 22 showed duplications on chromosme 16 (Figure 21).

- Patient 10 showed two duplications, respectively of 173 Kb and 164,22 Kb in size (arr[hg19] 16p11.2(32,573,813-32,746,861)x3) (arr[hg19] 16p11.2(33,107,015-33,271,235)x3).
- Patient number 22 showed a duplication of 1,8 Mb in size (arr[hg19] 16p11.2(31,986,098-33,786,268)x3).

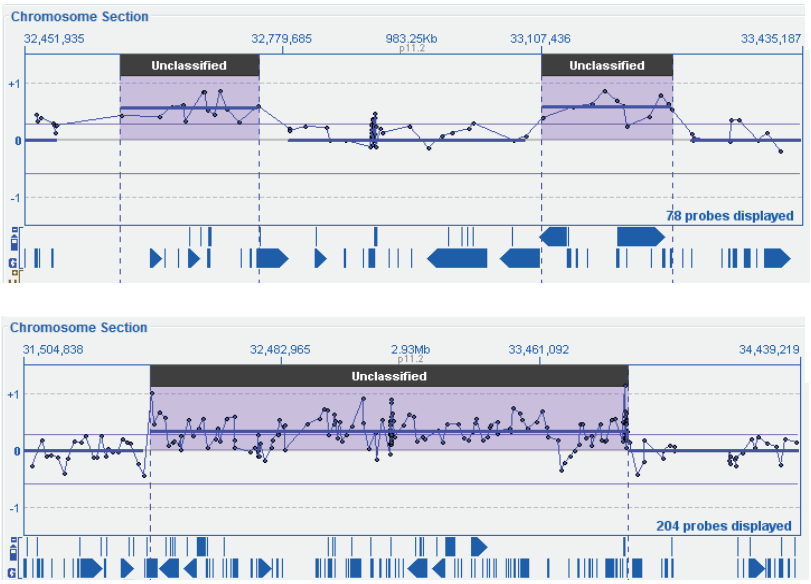


Figure 25 - Duplications on chromosome 16. Patient 10 is rappresented in panel A, patient 22 in panel B.

5 – DISCUSSION

To overcome the limits of classical karyotype and FISH analysis, the Comparative Genomic Hybridization array (aCGH) is widely used to identify genomic imbalances in both constitutional and neoplastic cells and has revolutioned the field of clinical cytogenetics.

Standard oncoematologic cytogenetics requires spontaneous dividing cells and, sometimes, results may be biased due to overcrowding of the cytogenetically normal cells compared to the abnormal ones. In addition cytogenetic analysis technique with QFQ/GTG banding has the limit of low resolution of about 10Mb.

FISH analysis can be applied to metaphases or interphases nuclei and it can be used not only to detect numerical changes or structural aberration. The search for abnormalities is not intended to study the entire genome, but only the regions of interest, for which specific probes are available and its application needs a specific diagnostic suggestion.

Array-based comparative genomic hybridization offers efficient high-throughput analysis of the entire genome for the identification of copy number variation/aberrations that occurr in clonal populations and would be undetected by conventional cytogenetic assays. It has improved resolution down to 100Kb. In addition SNP analysis is able to detect LOH/UPD (Loss of heterozygosity or Uniparental dysomic regions,) which are frequently involved in the cancer development.

We applied aCGG + SNP analyses on 22 AML patients analized by classical cytogenetics and with normal karyotype (NK-AML) in order to detect cryptic somatic imbalances and in 4 AML patients with complex karyotype in order to redefine the cytogenetic result.

Moreover, we performed aCGH on peripheral lymphocytes of a cohort of pediatric/adolescent patients that performed kidney transplantation, in order to investigate constitutional CNVs with possible predisposing significance to lymphoproliferative disorders development (PTLD) that occurs in 20% of these patients.

The purpose of these different studies is to detect by aCGH genomic alterations, acquired in AML or constitutional in PTLD patients, with possible oncogenic role.

AML with normal karyotype

About 50% of AML cases failed to show cytogenetic abnormalities (Akagi et al., 2008; Kim et al., 2012; Bullinger et al., 2009).
Therefore, one of the current challenges in the study of AML is to identify hidden or cryptic genomic lesions that may be related to AML origin and patient outcome. Array CGH with high oligonucleotides' density in genes or genomic regions involved in hematological malignancies has been used to identify submicroscopic copy number variants across the genome.

There are only few papers in literature about cryptic CNVs in NK-AML (Raghavan et al., 2005; Tyybakynoja et al., 2007; Akagi et al., 2008; Gupta et al., 2008; Bullinger et al., 2009; Kim et al., 2012; Spina et al., 2015)

Tyybakinoja et al., found cryptic CNVs in about 85% of NK-AML patients; Akagi et al. found CNVs and CNN-LOH in 49% of 30 NK-AML cases; Bullinger et al., studied 157 NK-AML patients and found CNVs in 49% of cases and CNN-LOH 12% of cases; Kim et al., studied 30 NK-AML patients in relation with treatment response and found CNVs in 76% of cases; Spina et al. studied 36 NK-AML patients and found CNVs in about 40% of cases.

Gupta et al., in 454 patients of AML with normal or abnormal karyotype and found non-random acquired LOH in 17% of cases. Raghavan et al., studied 64 AML patients and found CNN-LOH region in 18% of cases.

All together these studies suggest the clinical relevance and the prognostic significance of CNVs/LOH lesions in NK-AML and that aCGH- SNPs studies in larger cohorts of cases should improve prognostic stratification of patients and may reveal new therapeutic targets.

We performed a retrospective study by CGH+SNP array 60K Hematological platform on 22 samples of primary NK-AML at the onset of the disease.

Diagnosis of de novo AML was established at the Hematology Department of our hospital.

FAB classification is available for Patients 1 (M5), Patient 7 (M4), Patient 8 (M2), Patient 13, 17, 18 (M5) and Patient 22 (M0).

QFQ-banding cytogenetic analysis was carried out on at least 20 non-stimulated bone marrow metaphases in each case. The following cytogenetic controls during the evolution of the disease always confirmed persistent normal karyotypes.

Array CGH was applied to genomic DNA from bone marrow mononuclear cells and blasts, separated by Ficoll. Array CGH was also applied on cultured bone marrow-derived fibroblasts in cases with significant not polymorphic CNVs/LOH regions, in order to verify their somatic nature.

At this purpose, we excluded the CNVs that are common polymorphic regions (data not reported in table 5 and 6) or CNVs that does not contain cancer correlated genes (loss of 17q21.31, Patient 8). All the CNVs and LOH considered and discussed are not constitutional and they are present in 100% of cells, as indicate by Cytosure OGT analysis software, used in our laboratory.

The aCGH results confirmed the normal karyotype found by cytogenetic investigations in 50% of cases (11 out of 22) but in the other 50% of the cases detected additional significant aberrations not found by cytogenetic analysis, according to the data reported by Akagi et al., 2008 and Bullinger et al., 2009.

We found CNN-LOH in 5 out of 22 patients (about 25%). This rate is similar to data from literature (Akagi et al., 2008; Bullinger et al., 2009).

Genomic findings

In 50% of the case (11 out of 22 cases) CGH array analysis detected both somatic CNVs and /or LOH, which are variable in size from few Kb to several Mb.

In 5 cases array CGH unexpectedly revealed some relevant aberrations, such as aneuploidies +10 (Patient 4), +4 (Patient 6), +13 (patient 8), -Y (Patient 17), +8 and big structural rearrangements leading to trisomy of entire 1q (Patient 21), monosomy 7 , deletion of entire 5q, duplication of 16p and deletion of 16q (Patient 22).

These results are surprising because these aneuploidies are commonly dectected by cytogenetic analysis on leukemic bone marrow cells, but in this study a normal karyotype was diagnosed on at least 20 metaphases in all cases, both at onset and during the subsequent cytogenetic monitoring of the disease. Moreover, these changes are known to be characteristic of AML subtypes, and some of these (-7, -5q, +8, +13, -Y) have a specific diagnostic/prognostic and therapeutic value (Mitelman database).

The reason of the great discordant results found by cytogenetic and aCGH analysis is not clear.

One possible interpretation is that Ficoll-gradient applied on bone marrow samples used for DNA estraction to aCGH, selects mononuclear cells and blasts, thus CGH array was performed on selected cells that differs from the cell pool cultured for cytogenetic analysis.

Otherwise, another interpretation of this discrepancy, is that the lack of cytogenetic evidence of such abnormalities may be related to low mitotic index of such leukemic clones.

We cannot exclude an hypothetical influence of additional CNVs or LOH on the proliferation index of clones with aneuploidies or great imbalances.

We cannot confirm the results with interphase FISH analysis because in this retrospective study no material were still available.

Wrong interpretations of results by aCGH software analysis were excluded.

Trisomy 10 as sole abnormality (patient 4) is reported in 0.2% to 0.5% of AML cases but probably its frequency is underestimated. Trisomy 10 has been, however, described in all of the FAB AML variants except M3 and especially in M2 . Genes involved are unknown and the hematological and clinical features associated with this finding have not yet been clearly defined (Mitelman database; Kwong YL et al., 1993).

Trisomy 4 is another rare chromosomal abnormality in AML, occurring in less than 1% of cases. Although its prognostic relevance has been frequently debated, its association to outcome remains unclear, because of the rarity of trisomy 4 as an isolated abnormality (Chilton L et al., 2016).

Trisomy 13 is a rare chromosomal abnormality in AML with incidence rate of less than 1%. It has been reported that trisomy 13 is strongly associated with presence of RUNX1 mutations and a high expression of FLT3 mRNA correlated to poor prognosis (Silva FP et al., 2007).

Trisomy of chromosome 8, monosomy 7, monosomy 5q, loss of sexual chromosomes are, on the contrary, often involved in AML (Mitelman database) and of well defined prognostic significance.

About the structural abnormalities found, the duplication of all or part of the long arm of chromosome 1 (Patient 21) is one of the most frequent chromosomal abnormalities in human neoplasia. It has been reported in patients with various myeloproliferative conditions, including AML. Although cytogenetically heterogeneous, duplication of the 1q21-1q32 segment is most commonly observed, indicating that certain chromosome 1 regions might harbor genes implicated in oncogenesis. Among them, the expression of CKS1B gene is elevaled in multiple cancer and is associated with poor prognosis.

Gain of chromosome 1q is likely implicated in neoplastic processes by a gene dosage effect. Whether appearance of 1q duplication may be sufficient as the sole anomaly to promote leukemogenesis is unclear, as well as it's role in cytotoxic therapy. Patient 21, also showed a molecular mutation of NMP1 gene. All these anomalies taken together probably contributed to resistance to chemotherapy, although NMP1 gene is a favorable prognosis marker

Trisomy of entire 16p and a monosomy of entire 16q are present in patient 22 togheter with the others abnormalities (-7, -5q, loss 4q32.1q34.3, loss 12p13.2p12.1). Total or partial trisomy/monosomy of chromosome 16 are not currently described in AML cases, but may be a new cryptic abnormality. Patient 22 showed a very complex molecular karyotype: in addition to monosomy 7, monosomy 5q, trisomy 16p and monosomy 16q, the patient showed the deletion of 12p13.2p12.1.

Cytogenetic abnormalities involving the short arm of chromosome 12 have been documented in a wide variety of hematopoietic malignancies, including AML (Kobayashi, H et al., 1994)The reported deletion encompass the CDKN1B and ETV6 genes. A minimal interstitial deletion region is described, involving ETV6 and CDKN1B genes;

CDKN1B, is a cyclin-dependent kinase inhibitor that blocks the cell cycle in the G0/G1 phase upon differentiation signals or cellular insult. CDKN1B also regulates cell motility and apoptosis (Cuesta R et al., 2009). Homozygous deletion of CDKN1B is rare (Mietelman Database)

ETV6 is frequently deleted in hematological malignancies; the deletion of the normal (untranslocated) ETV6 allele in the presence of a translocation affecting ETV6 is quite frequent, notably in patients with ETV6-RUNX1, ETV6-NTRK3, ETV6-ABL1, ETV6-ACSL6 and ETV6-STL fusion. Deletion of an ETV6 allele has also been observed in the absence of rearrangement of the second allele (Mitelman database). Hemizygous interstitial 12p deletion is found in 10% of cytogenetically normal AML (Mitelman database, Feurstein S et al., 2014; Andreasson P et al., 1997)

The deletion of 17 Mb on Chromosome 4 seems to be not involved in specific onco-hematological events, but all these finding taken together are probably the cause of the resistant to therapy of the patients.

About LOH findings, in two cases (Patient 6 and 8) the same LOH of the quite entire q arm of chromosome 11 is present, as additional aberration to trisomy +4 and +13, respectively. LOH on 11q is one of the most recurrent finding in myeloid malignancies (Dunbar et al., 2008). Among several candidate genes on 11q the proto-oncogene, CBL is relevant in the pathogenesis of AML. Mutation in exons 8/9 of CBL gene plays a role in human FLT3 downstream signalling (Sargin B et al., 2007) and homozygous CBL mutations were found in most 11q acquired UPD-positive myeloid malignancies.

11q13.2q25. MLL gene, located on 11q23.3, is an important player in epigenetic regulation in AML (Jin S et al., 2010) and the N-terminal part of the MLL-protein is fused to more than 70 possible fusion partners (Tamai et al., 2010). A LOH in 11q encompassing MLL gene was reported also in Gupta et al.

Patient number 2 showed a LOH of chromosome 2p, and no other chromosomal abnormalities or genic mutation: the meaning of this loss of heterozygosity in unclear. Also Gupta et al., identified LOH on 2q, suggesting that this region might be a novel candidate region involved in AML(Gupta et al., 2008).

CNVs and LOH regions may have opposite effects on cells proliferations and patients' outcome: Patient 6 with +4 and LOH 11q was resistant to therapy, Patient 8 with +13 achieved a complete remission. This interpretation of results need to be expanded in a wider range of case studies.

Some cases showed, as only genomic abnormality, little CNVs: gain 3q26.32q26.33 (patient 1), loss 7qq22.1 (patient 2), gain 4p16.3, gain 7q22.1, gain 17q21.31 (patient 17), loss 4q32.1q34.3 and loss 12p13.2p12.1 (patient 22).

Patient 1 showed a gain 3q encompassing PIK3CA gene, that provides instructions for producing the p110 alpha (p110α) protein, which is one subunit of an enzyme called phosphatidylinositol 3-kinase (PI3K). PI3K signaling is important for many cell activities, including cell growth and division (proliferation), movement (migration) of cells, production of new proteins, transport of materials within cells, and cell survival. PIK3CA is a transforming oncogene that was shown to have activating mutations in the commonly occurring cancers (Kang S et al., 2005).

Patient 2 showed a cryptic loss on 7q22.1 of 41 Kb. This deletion encompasses CUX1 gene, which is normally highly expressed in multipotent hematopoietic progenitors and exhibits dynamic dosage changes during the course of differentiation.

CUX1 gene encodes a homeodomain-containing transcription factor that regulates cell cycle progression and apoptosis.

Deletion of a single allele of CUX1 is associated with haploinsufficiency transcript and protein levels (Megan E et al., 2013).

This gene is tumor suppressor gene, frequently deleted in myeloid neoplasms (Megan E et al., 2013).

Two patients (Patient 1 and 2) showed NPM1 mutation. PIK3Ca duplication and CUX 1 deletion seem to have a negative prognostic significance, in contrast to positive meaning of NMP1 mutations. Both patients relapsed and we can assume that the effect of the altered gene dosage of PIK3Ca and CUX1 is prevalent on NPM1 mutation and plays a role in the outcome of the disease.

Patient 10 showed a non significative gain in 10q25.1; this region does not contain gene involved in AML development.

Patient 17 showed a 7q gain overlapping the just mentioned haploinsufficient CUX1 gene, a gain 4p encompassing FGFR3 and a gain 17q encompassing FMNL1.

FGFR3 is constitutively activated in a large portion of epithelial, bladder and cervix cancers (Cappellen, D et al., 1999) but its role in leukemia has not been estabilished yet. Moreover, patient 17 showed a gain in FMNL1 gene, located on 17q. This gene is over-expressed in a variety of hematopoietic malignancies (Favaro et al., 2003; Favaro et al., 2006; Schuster et al., 2007).

The overexpression of FGFR3 and CUX1 may have an irrelevant role in the progression of the disease, as shown by the complete remission of this patient. The correlation of triplication of FMNL1 gene and complete remission of the patient remains unexplained.

There are not significant differences about clinical outcome (remission, relapse, theraphy response) between the group of patients without CNVs/LOH and patients with several CNV abnormalities. This may be due to the limited number of cases analyzed and probably to the fact the therapy was based on the finding of normal karyotype or molecular mutations. It is not possible to exclude that a different therapy approach, based on the emerging cryptic abnormalities detected by aCGH, could modify the clinical evolution of the disease.

Inspite of the absence of clear prognostic implications, this study suggests the utility of aCGH analysis at onset of NK-AML patients because it may reveal unespected genomic abnormalities confined in cryptic clones, possibly influencing the therapheutic choises.

Array CGH investigation reveal it's great utility in 4 additional AML cases in wich cytogenetics identified complex chromosome aberrations not well definibles or absence in mitotic index.

In patient 1 (group 2 table) aCGH array revealed two submicroscopic deletions 17p11.2-p11.1 and 17q21.1 in a APL case that allow to define the rare APL variant with STAT5B-RARA fusion gene. As this rare variant shows resistance at the classical ATRA –ATO therapy, the identification by array-CGH was of high clinical relevance.
Acute promyelocytic leukemia (APL) is a rare subtype of acute myeloid leukemia (AML) and constitutes 5-8% of all AML cases. About 98% of APL patients exhibits the specific chromosomal translocation t(15;17)(q24;q21) involving the promyelocytic leukemia gene (PML) on chromosome 15q24 and the retinoic acid receptor alpha gene (RARA) on chromosome 17q21, resulting in an aberrant fusion gene PML-RARA. The hybrid protein inhibits the cellular differentiation and promotes an uncontrolled myeloid precursor cells proliferation (Pessina C et al., 2016, Rowley JD et al., 1977; Chen Z et al., 1992; Mistry AR et al., 2003; Grimwade D et al., 2000).

Few patients with APL morphology present variant translocation involving RARA gene. The alternative partner genes include: ZBTB16 (zinc finger- and BTB domain-containing protein 16, 11q23 chromosome band), NUMA1 (nuclear mitotic apparatus protein 1, 11q13), NPM1 (nuclephosmin, 5q35), FIP1L1 (factor interacting with PAPOLA and CPSF1, 4q12), PRKAR1A (protein kinase A regulatory subunit type 1A, 17q24), BCOR (BCL6 corepressor, Xp11), and STAT5B (signal transducer and activator of transcription 5 beta, 17q21) (Pessina C et al., 2016).

The variant cases characterized by ZBTB16-RARA and STAT5B-RARA fusions genes are notably resistant to ATRA (Melnick A et al., 1999) STAT5B-RARA is the most rare type of fusion transcript described so far with only 8 reported cases (Chen H et al., 2012; Melnick A et al., 1999; Arnould C et al., 1999; Gallagher Re et al., 2004; Kusakabe M et al., 2008; Iwanaga et al., 2009;Qiao et al., 2011; Jovanovic et al., 2011, Wang et al.,2015) two of wich showed the same del 17q.

This case suggest that a complete molecular analysis, including array CGH, is warranted in case of a morphological diagnosis of APL without the classical PML-RARA.

In another APL patient (case 2- group 2) cytogenetic analysis showed a balanced translocation involving chromosome 4 and 17. CGH array, consented to better define the complex karyotype, permit to exclude the suspected involvement of chromosome 11 in the balanced translocation and to identifie a deletion 2p14-p11.2 of 15,726 Mb in size, not showed by conventional karyotype.

Array CGH is of relevant utility also when mitotic index is low and cytogenetic failed. Our patient 3 (group 2) was affected by Blastic Palsmacytoid dendritic cell neoplasm, that is an aggressive hematopoietic malignancy AML type, derived from the precursor of plasmacytoid dendritic cells. There are no formal studies on the incidence of BPDCN in the general population. The few available data reported indicate that its overall incidence is extremely low, accounting for 0.44% of all hematologic malignancies (Bueno C et al., 2004).This tumor is characterized by a high frequency of cutaneous asymptomatic lesions at diagnosis. Predominance genomic losses affects chromosome 5q21, 5q34, 12p13, 13q, 6q23, entire chromosome 9 (Leroux et al., 2002).

Cytogenetic analysis showed only few chromosome abnormalities, such as monosomy 9 and monosomy13 (according to literature data).
On the contrary Oligo-array technique defined a very complex genomic status with multiple rearrangements involving different chromosomes and reported in table 9, and Chromothripsis of chromosome 7.

Chromothripsis is a recently described phenomenon identified in cancer cells that produce catastrophic chromosome reorganization of one or a small number of chromosomes; it is been recently demonstrated that Chromothripsis generates marker chromosomes in AML and this phenomenon is associated with poor prognosis (Bochtler T et al Blood 2017, Jan 24).

Patient 4 had already shown cytogenetic abnormality on chromosome 5q and CGH array helped to better define the deletion. As expected, tetraploidy and the balanced translocation t(15;17) did not appear.

As a whole, CGH array was clinically and biologically useful, as in two cases it helps to identify the rare APL variant with therapeutic conseguences and in one case to identify complex abnormalities and chromotripsis phenomenon, which was not visible because of the lack of proliferation of leukemic cells.

This highlights the importance of arrays in non-proliferant clones and also in complex cases.

Inspite of the limited number of cases, this study suggests the clinical utility of aCGH at onset of the disease, in AML patients with Normal karyotype and in patiens with low mitotic index or complex karyotype.

Microarrays do not replace the conventional cytogenetic testing methodologies because they cannot detect truly balanced rearrangements or clones less than 20% of the cells, but it is evident that the incorporation of microarray testing into the routine clinical diagnosis of AML with normal karyotype at the time of diagnosis could be the right strategy required to enhance interpretation of the disease.

Post- transplanted patients

The second goal of this thesis through CGH array investigation is to identifie possible germline copy number imbalances (Constitutional CNVs) in pediatric patients tht performed kidney transplantation, in order to detect constitutional CNVs possibly correlated or predisposing to development of PTLD (post transplantation lynphoproliferative diseases).

PTLD (PT-large B cell lymphoma, PT-DLBCL; PT-Burkitt lymphoma PT-BL; plasmoblastic lymphoma, PT-PBL) are a serious complication and appear in about 20% of transplanted patients.

EBV infections or its reactivation and immunosuppression are considered as predisposing factors, nowadays, there are no data regarding the finding and the impact of constitutioal CNVs on the development of PTLD.
A cohort of 23 pediatric/adolescent patients kidney transplanted for various disease, such as Nephronophthisis juvenile and renal displasia/hypodisplasia, were explored in order to investigate constitutional CNVs.

Genomic DNA was taken from pre-transplantation T-lymphocytes.

Patients were divided into four groups:
- group 1: patients that developed PTLD (number: 10, 11, 12, 13, 14, 15, 16, 17);
- group 2: patients that do not developed PTLD and have EBV- High Viral Load. This group has a high risk of developing lympoproliferative disease (number: 4, 9, 20, 22);
- group 3: patients without PTLD and with EBV Low Viral Load (number: 5, 7, 19,);
- group 4: patients with absence of clinical manifestations (number: 1, 2, 3, 6, 8, 27, 30).
These two last groups are considered as controls.

Clinical data and EBV-status were reported in table 9.
We detected many CNVs, all reported in table 11.
Table 12 illustrates recurrent CNVs found in the four different groups of patients.

The total number of detected CNVs found in Controls/Low Viral load patients (first/second group) and High Viral load/PTLD (third/fourth group) do not show any significant difference.

CNVs exclusively or mostly present in PTLD/EBV high viral load (PTLD-HV) group are:

Duplication on chromosome 1p21.1

This duplication is present in 7 out of 22 cases, not-equally spread in the four patients' groups, as shown in Table 12.
The duplication is present in 6 out of 12 patients (50%) of third/fouth group and only in 1 out of 10 patients (10%) of the control and low viral load groups.

Patient 14 shows a constitutional Gain of 776Kb, disprupting by its start point, the COL11A1 gene, suspected to be involved in carcinogenesis (Li et al., 2017).

The other 5 cases (number 20 and 22 of Third group; number 10,11,16 of PTLD group and the control case number 2) show a smaller duplication of about 100Kb that include the polymorphic AMY-gene family but exclude COL11A1 gene.

Deletions and duplications 6p25.3

These small CNVs involve the DUSP22 gene, recently described as tumor soppressor gene in T-cell lymphoma subtypes (Mélard P et al. 2016). Four cases of High Viral Load and PTLD patients, present both Gain (number 22,15) and Loss (number 9,20) of this region.
In all cases DUSP22 gene is disrupted by the end-point of the CNV, in its initial region.
This finding let us suppose a possible predisposing significance of this gene in the development of PTLD. However we can note that 3 out 4 patients nowadays have not yet developed PTLD, in spite of they present a High Viral Load.

Duplication and Deletion on chromosome 14q32.33

These CNVs are only present in PTLD patients and they are recurrent in B cell malignancies. (Takashima et al., 1997; Urbankova et al., 2012). Our interesting finding let us suppose that both duplications and deletions of this chromosomal band constitutional predispose to lymphoproliferative disorders.

Duplication on chromosome 16p11.2

This aberration is present in 2 case: patient 22 is a High Viral Load and patient 10 is a PTLD.
The duplication of this region is a genetic abnormality frequently observed in pediatric B cell lymphomas, DLBCL (Deffenbacher et al., 2012).

In these cases, the gene involved in duplication is TP53TG3, that is involved in TP53-mediated signaling pathway. Considering the nature of TP53 gene, a genomic imbalances of genes correlated to TP53 pathway, let us suppose a possible role in cancer predisposition.

Patients number 1 and 13 present an homozygous deletion of chromosomal region 2q13, encompassing NPHP1 gene, whose homozygous deletion is causative of 75% of Nephronophthisis juvenile, pathology affecting both two patients (OMIM numbers: 607000 and 256100 respectively). Patients 14 also presents a deletion in 2q13, but NPHP1 gene is excluded.

Cases are spread equally in the two groups and do not correlate with PTLD.

The results of this study, inspite of the limited casistic, let us make few consideration:

CNVs containing oncogenes (Col11A,DUSP22,14q32, TP53TG3,or CNVs described in lynphoproliferative disorders are exsclusively or most frequently found in gropups of patient tha developed PTLA or with high viral load so at risk to develop it.
The presence of the same CNVs in more than one group of patients suggest the probably necessity of a second genetic or environmental hit to start the neoplastic transformation and PTLD development.

It will be interesting in the future to follow up cases with the same CNVs and absence of clinical manifestations, but with different Viral load.
More case necessitate to conferme this suggestions.

In conclusion our work confirm that array CGH is superior to other methodologies in detecting somatic chromosome aberrations not found with cytogenetics in primary AML defining their biogical and clinical significance and in exactly defining complex rearrangements with a single experiment. In addition, aCGH reveals constitutional CNVs that may be predisponent to PLTLD development.

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ABSTRACTS

VARIABLE EXPRESSION OF SHOX AND KANK1 GENES IN A FAMILY CASE

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The short stature homeobox-containing gene (SHOX) is located in the pseudoautosomal region1 (PAR1) of both sex chromosomes and it escapes from X-chromosome inactivation. SHOX gene alterations are associated with a wide phenotypic spectrum such as Idiopatic Short Stature, Leri-Weill Dyschondrosteosis and Langer Syndrome with variable penetrance also in the same family. Recently, SHOX duplications seem to be also correlated with Intellectual Disability (ID).

KANK1 gene is located at chromosome 9p24.3 and it encodes a protein involved in cytoskeleton formation by regulating actin polymerization. Mutation in this gene have been associated so far with cerebral palsy, Spastic Quadriplegia type 2 and central nervous system development disorders. KANK1 deletions have been described in cases with variable phenotype, such as Autism Spectrum Disorder, motor delay, ID and short stature.

Here we describe the first family case with SHOX deletion and 9p24.3 microduplication. We performed Conventional cytogenetics analysis, array CGH and MLPA of SHOX gene. We detected a 592 Kb deletion in Xp22.33 and a 279 Kb duplication in 9p24.3 including KANK1 gene. The deletion encompasses the entire SHOX coding region and also part of the regulatory upstream elements, while the start point of duplication is located within KANK1 gene. Since KANK1 has many alternative splicing sites, we can't leave out that this CNV may alter the expression of some transcripts.

Father is carrier of both alterations and he has trasmitted SHOX deletion to all children while 9p24.3 duplication only to 2/3. In this family SHOX gene deletion, as previously described in the literature, crossovers between X and Y chromosome during paternal meiosis resulting in an unusual inheritance. All family members with SHOX deletion present short stature or growth delay. KANK1 deletions are associated with dysmorfic features, ID, delay/absent speech and motor developmental. In our case, only one child shows such phenotype: we suppose an incomplete penetrance effect of KANK1 duplication, and that SHOX deletion together with KANK1 duplication may result in more severe phenotype. Anyway, phenotype-genotype correlation will be more clear during pubertal age, when clinical features are well define.

A NEW CASE OF MAYER-ROKITANSKY-KUSTER-HAUSER TYPE 2 SYNDROME WITH del(17)(q12). GENOTYPE- PHENOTYPE CORRELATION AND LITERATURE REVIEW

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Mayer-Rokitansky-Kuster-Hauser (MRKH) syndrome is characterized by anomalies of genital tract ranging from upper vaginal atresia to total mullerian agenesis with urinary tract abnormalities (OMIM 277000). It's incidence is about 1/5.000 newborn females. Affected women show 46,XX normal karyotype. MRKH may be isolated (MRKH Type 1- OMIM 277000) and comprises CAUV (Congenital Absence of Uterus and Vagina) and mullerian aplasia, or associated, in about 40% of MRHK patients, with renal, skeletal (mainly vertebral) malformations, hearing defects and, more rarely, cardiac anomalies (MRKH Type 2 or MURCS association- Mullerian duct aplasia, Unilateral renal agenesis and Cervicothoracic somite anomalies, OMIM 601076; GRES - Genital, Renal, Ear, and Skeletal when applied to both sexes). During last years, many cases of MRKH Type 2 and additional altered phenotypes have been published. The additional clinical traits are different and the most recurrent are MODY5 (Maturity-Onset Diabetes of the Young Type 5 OMIM 137920), facial dysmorfisms, moderate intellectual disability. Gene point mutations in CFTR, WNT4, TBX2, LHX1, HNF1B genes, and few genomic im- balances (CNVs) particularly 17q12 and 16p11.2 losses, are reported.

We describe a 17 years old female patient with a MRKH type 2 complex phenotype consisting in a new, not previously reported association of MRKH Type 2, Autism Spectrum Disorder (ASD), moderate intellectual disability (ID) and psycho- motor delay. A 1,74 Mb 17q12 microdeletion, including 30 OMIM genes and MIR2909 gene, was identified by array CGH investigation. We review the literature and discuss the role of the del(17)(q12) in the MRKH phenotype.

p.Gly130Val MUTATION IN THE GJB2 GENE. A FAMILIAR DOMINANT HEARING LOSS WITHOUT SKIN MANIFESTATION

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Connexins are a multigene family proteins building gap junctions, intracellular channels found in all human body tissues, including nervous tissue, heart, muscle and skin. Several form of hearing loss are due to connexins mutations, but it has been demostrated that connexins are also involved in regulation of growth and differentiation of epidermis, so that many hereditary disorders, affecting both the epidermis and cochlea, have been linked to connexins mutations.

In particular mutations of GJB2 gene (gap junction protein beta2), located on chromosome 13q12 and encoding the con- nexin 26 (Cx26), are involved in hearing loss diseases associated with skin problems such as Keratitis-ichthyosis deafness syndrome (KID), Palmoplantar Keratoderma with deafness (PPK), Hystrix-like ichthyosis deafness (HID), Vohwinked syn- drome, Bart-Pumphrey syndrome.

Here we report a family (father and son) with sensorineural hearing loss, carrying heterozygous p.Gly130Val mutation in GJB2 gene but, for the first time, without any dermatological problems. Only other two families in literature reported the same mutation, but associated with skin abnormalities. p.Gly130Val is located on the second domain of GJB2 gene and it has been reported in patients with Vohwinkel syndrome, an autosomal dominant disorder characterized by congenital deafness, hyperkeratosis of the soles and palms, constrictions on the fingers and toes. Proband is a 6 years old male with post verbal congenital bilateral profound sensineural form of deafness, identified clinically at 3 years of age. His father have analogous phenotype, while the other family members (brother and paternal grandparents) don't show neither mutation in GJB2 or hearing loss. We screened other genes involved in deafness (GJB6-d13s1830, GJB6-d13s1854, Mir-96, GJB3, GJA1, A1555G and A7445G) but no mutation were found. GJB2 mutations associated with additional skin abnormalities have dominant inheritance pattern and very heterogeneous clinical features, also in the present family although there are no dermatological problems. We can suppose that skin manifestations may be due to additional mutations or gene modifiers. Environmental factors during development or genetic background may also have a effect on phenotypic variation.

SHORT STATURE AND isoYp CHROMOSOME. A CASE WITH THREE COPIES OF SHOX GENE

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Short stature homeobox containing gene (SHOX) is located in telomeric region of both sex chromosome, in pseudoautosomal region 1 (PAR1). SHOX is one of the most important gene involved in scheletal growth and it is studied in patients with idiopatic short stature (ISS). The function of SHOX is dose depend: haploinsufficiency causes ISS and Lery Weill Dyscondrosteosys while triple dose of gene it seems linked to tall stature as in Klinefelter patients. However some cases with short stature and SHOX and/or regulatory elements duplications have been reported in literature. Authors have suggested that in these cases there is the lack of interaction between gene and regulatory sequences.

Here we describe a 15 years old boy with three copies of gene resulting from a isoYp chromosome. The patient is the only child of non consanguineous parents. He shows short stature (146 cm, <3%ile,-2SD) with normal values of GH (growth hor- mone), recurrent otitis, hearing loss, mild gynecomastia, normal external genitalia and spontaneous pubertal development. Cytogenetic analysis and FISH, performed in other centre, showed a derivative Y chromosome in all 100 metaphases analyzed. The final karyotype is 46,X,psu idic(Y)(pter->q12::q12->pter).ish psu idic(Y)(q12)(SRY++,DYZ1-). MLPA analysis of SHOX (P018-G1) confirmed the presence of three gene and nearby region copies. Furthermore, control probe located in the PAR2 (pseudoautosomal region 2) in q arm of both sex chromosome has resulted in single copy to demostrate the loss of Yq. No point mutations have been identified in coding regions by Sanger sequencing.

Here we want to demonstrate the variable effect of triple dose of SHOX gene on final stature. In this patients gene and regulatory sequences are completely triplicate. We suppose that other gene on Yq or regulatory sequences are involved in the final phenotype. Array CGH analysis will help us to identify how many and which genes are deleted on Yq and will better define the break point in Yp.

Studio collaborativo GdL SIGU Cito-genetica/-genomica: risultati preliminari relativi a 4142 diagnosi postnatali effettuate mediante CMA (Chromosomal Microarrays Analysis)

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Lo studio, condotto dal Gruppo di Lavoro SIGU di Citogenetica e Citogenomica, ha visto la partecipazione di 12 laboratori
italiani che in totale hanno eseguito 4142 analisi postnatali mediante CMA (Chromosomal Microarrays Analysis). Ne vengono qui presentati i risultati preliminari, ricavati da una prima rielaborazione dei dati raccolti.
L'indicazione all'analisi è stata: ritardo dello sviluppo nel 17% dei casi e associato a dismorfismi nel 2%; sola disabilità
intellettiva nel 12%, associata a dismorfismi nel 9%, a malformazioni nel 5%, a epilessia nel 4%; autismo nell'11%; presenza
di sole malformazioni nell'8%; sospetta sindrome da micro-delezione/-duplicazione nel 7%; presenza di soli dismorfismi
nel 3% e di sola epilessia nel 2% dei casi. Nel restante 20% dei casi le indicazioni erano rappresentate dalla combinazione
di più segni clinici.
Il cariotipo molecolare è risultato normale o con presenza di sole CNVs (Copy Number Variations) benigne in 2770 (67%)
soggetti indagati ed anomalo in 1372 (33%). Le CNVs riscontrate, escludendo le benigne, sono state complessivamente

1744 in quanto alcune presenti contemporaneamente in uno stesso paziente. Di queste, il 33% è stato interpretato come
clinicamente rilevante, l'11% come VOUS (Variants Of Unknown Significance) probabilmente patogenetica, il 25% come
VOUS probabilmente benigna ed il restante 31% non è stato possibile classificarlo. Escludendo le categorie di indicazione
all'analisi con un numero di pazienti indagati inferiore a 100, la percentuale più elevata di cariotipi molecolari anomali è stata
osservata nei soggetti inviati per: dismorfismi (47%); sospetta sindrome da micro-delezione/-duplicazione (40%); ritardo
dello sviluppo (33%) e associato a dismorfismi (38%); disabilità intellettiva associata a malformazioni (38%), a dismorfismi
(34%) o ad epilessia (28%), malformazioni (31%), sola disabilità intellettiva (27%); autismo (25%).
L'elaborazione finale dei dati consentirà di trarre ulteriori informazioni e di raffrontarle con quanto riportato in analoghi studi
presenti in letteratura

PUBBLICATION

Pessina C, Basilico C, Genoni A, Meroni E, Elli L, Granata P, Righi R, Pallotti F, Mora B, Ferrario A, Passamonti F, Casalone R. "A new acute myeloid leukemia case with STAT5B-RARA gene fusion due to 17q21.2 interstitial deletion". Leukemia and Lymphoma. 2016 Dec 2:1-4.