University of Siena

Ph.D in Medical Genetics

Genomic deletions in patients with complex phenotypes: from cytogenetics to array-CGH.

Chiara Pescucci

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Thesis suitable for the title of “Doctor Europeus”

Academic year 2005-2006
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Siena May 28, 2006

Please receive the Ph.D thesis of Chiara Pescucci.

She started the Ph.D course in Medical Genetics in October 2001, after a degree in Biological Science. During these years she carried out several lines of research which include Alport syndrome (Pescucci et al. Kidney Int 65:1598-603, 2004), glaucoma, Rett syndrome and X-linked mental retardation. In this latter field she helped me in writing the N&V for Nat Genet: Is Rett syndrome a loss-of-imprinting disorder? Nat Genet. 2005 Jan;37(1):10-1.

She chose to discuss the final Thesis on patients with mental retardation and complex phenotype and their characterization by array-CGH since this technique has represented in the last year a revolution in Medical Genetics filling the gap between Cytogenetics and Molecular Genetics.

Starting from this year, it is possible in Italy to get a title of “Doctor Europeus”. This title can be conferred by the University of Siena, which is one of the Italian pioneer Universities in this field, when the following criteria are fulfilled:

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I ask you to be the external reviewer of this thesis. Please send me back your general opinion together with specific comment whenever necessary.

Sincerely,

Prof. Alessandra Renieri
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Curriculum Vitae and List of Publications
with love and curiosity.
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First of all, I would to thank prof. Alessandra Renieri for her continuous and precious activity as teacher and supervisor of my work. If someone ask to me to define this “PhD years”, I think that the best words should be “exciting and hard”.

I would like to thank prof. Orsetta Zuffardi and coworkers for the important technical and cultural support and dr. Manuela Priolo for her smart contribution to the discussion of clinical cases.

A special though to my colleagues…..I shared with them an incredible series of scientific and personal adventures….

Finally, I would like to dedicate this “microscopic” step towards knowledge to children and their families, because the protagonists of Medical Genetics are persons and not techniques.
1. INTRODUCTION
1. INTRODUCTION

1.1 Conventional cytogenetic analysis of chromosomal rearrangements

The term cytogenetics is conventionally referred to the study of number, structure and evolution of chromosomes. Cytogenetic techniques allow the identification of numeric and structural chromosomal aberrations that cause diseases. The first drawn illustration of chromosomes was published in 1882 and the term “chromosome” was introduced in 1888. Conventional chromosome analysis was developed from the first years of 1950s. During these years several discoveries were made. In 1952, Hsu et al. described the “hypotonic shock” in cultured cells. He discovered that cells exposed to an hypotonic salt solution for several minutes prior to fixation procedures gave a much better chromosome spreading. In 1956, Ford introduced the use of colchicine in chromosome preparation. This substance acts as a “mitotic poison” and allow to capture cells in their metaphase stage, increasing the number of metaphases available for microscope analysis. In 1956, the application of these technical improvements allowed to establish that the correct number of chromosomes in the human diploid cells is 46. During this new era of cytogenetics, although chromosomes showed only few details, they could be arranged in different groups on the basis of their size and centromere position. Through the application of these techniques the first numeric aberrations (aneuploidy) causing diseases in man were identified. In 1958, Lejeune et al discovered that patients with Down syndrome show a supernumerary chromosome 21 and, subsequently, the numeric aberration causing Klinefelter (47, XXY) and Turner (45, X) syndromes were identified. In the same period, the first acquired chromosomal anomaly (Philadelphia chromosome) was described in patients with chronic myeloid leukaemia. Subsequent technical improvements in cytogenetics were the use of phytoemoagglutinin and the introduction of banding techniques at
the end of 1960s. Phytoemoagglutinin is a substance that stimulates the division of T lymphocytes in vitro and allows the obtaining of chromosomal preparations from peripheral blood samples. Banding techniques use chemical treatments to produce differentially stained regions on chromosomes, producing patterns of dark and light bands along the length of each chromosome 10. The banding pattern is highly characteristic for each chromosome and permits the complete identification of the human karyotype. Several banding techniques were developed, each having their own specific properties and applications 1. The G-banding technique is based on the application of trypsin, a proteolitic enzyme, followed by Giemsa staining and it is the most diffuse method for routine use in a clinical setting (Fig. 1). Conventional cytogenetics became a powerful diagnostic tool to detect genomic aberrations, including both gains and losses of portions of the genome as well as rearrangements within and among chromosomes. However, the resolution of standard cytogenetics techniques remains relatively limited with an approximate count of about 400-500 bands per haploid genome. These approaches allow the identification of structural chromosomal aberrations of at least 5-10 Mb in size. Average resolution depends on different elements such as the optical characteristic of the microscope, the complex manner in which DNA is packaged into chromosomes and the quality of metaphasic preparations. The resolution of the standard karyotype was improved by the introduction of high resolution banding, based on the use of synchronized lymphocytes cultures 11. In this way, it is possible to increase the number of cells blocked in pro-metaphase or prophase stage. In these stages chromosome are much longer and the resolution increases up to over 1000 bands per haploid genome 1.
Fig. 1 | **GTG-banded karyotypes.** Standard human karyotypes at a resolution of about 400 bands per haploid genome. A) Normal female karyotype (46,XX); B) Normal male karyotype (46,XY).
1.2 Molecular cytogenetics

Molecular cytogenetic is the study of genetic disorders by the use of new techniques that combines cytogenetic and molecular approaches obtaining a great improvement in resolution. The first technique that has initially reduced the distance between cytogenetics and molecular genetics is FISH (Fluorescent in situ Hybridisation), developed in the first years of 80s. In this approach, the fluorescent labelled probe DNA is hybridised to a slide of metaphase chromosomes obtained from the patient. After wash procedures, that remove not bound DNA, slides are observed in fluorescent microscopy.

The sequencing of the human genome has generated an extremely important resource of mapped and sequenced clones, which are revolutionising molecular cytogenetics. Information on chromosomes are stored and available on several web based databases (Ensembl, http://www.ensembl.org/Homo_sapiens/cytoview; UCSC Genome Bioinformatics Site, http://genome.cse.ucsc.edu; MapViewer (http://www.ncbi.nlm.nih.gov/mapview/). Clones can be selected and obtained for use in FISH investigations of aberrant chromosomes and breakpoint spanning clones can be easily identified. However, in FISH investigations each probe have to be hybridised and then microscopically analysed. Breakpoints characterisation requires several rounds of hybridisations, starting with clones relatively widely spaced followed with clones at increasingly higher densities. These studies are labour intensive, time consuming, difficult to automate and can be applied for only a limited number of chromosomal loci at one time. Moreover, this technique requires prior knowledge of the type and location of chromosomal aberrations. Hence, new techniques to overcome these limits were explored.

In 1992, Kallionemi et al introduced a new chromosome analysis technique called Comparative Genomic Hybridisation (CGH) that allows a global overview of genomic gains and losses in a single experiment on chromosome spreads. Finally,
in the last years an important technological goal was reached with the introduction of array-based Comparative Genomic Hybridisation (array-CGH)\textsuperscript{16, 17}, a technique that has definitively fill the gap between cytogenetics and molecular genetics.

1.2.1 Comparative Genomic Hybridisation

Comparative Genomic Hybridisation (CGH) was developed in the early 1990s\textsuperscript{15}. CGH is a method designed for identifying chromosomal segments with copy number aberration and allows a genome-wide screening in a single experiment. In CGH total genomic DNA obtained from control cells and from a test sample is differentially labelled with fluorochromes, co-precipitated in presence of blocking DNA to suppress repetitive sequences and subsequently co-hybridised to normal metaphase chromosomes (Fig.2A). In the classic experimental approach, test DNA is labelled in green and control DNA (reference) is labelled in red. After hybridisation and washing procedures, metaphase spreads are observed by a fluorescent microscope and image analysis is performed with dedicated software. The resulting ratio of the fluorescence intensities of the test and the reference hybridisations are digitally quantified along the length of each chromosome. Chromosomal regions equally represented in both the test and the reference samples appear yellow for the presence of an identical amount of red and green fluorochromes, and have a ratio of 1.0. Regions of copy number gain appear green and have a ratio above one, while regions of copy number loss are red with a ratio below one (Fig.2 B). In this way, a global evaluation of copy number variations throughout the whole sample is obtained in a single experiment without the requirement of dividing cells\textsuperscript{18} (Appendix 1)\textsuperscript{19}.

CGH has been applied for the study of human diseases, given that gene dosage variations occur in many conditions from cancer to developmental abnormalities. Thus, detection and mapping of copy number abnormalities provide
an approach for associating aberrations with disease phenotype and for localizing critical genes. However, it is important to note that CGH detects only copy number variation, not absolute copy number. Whole-genome copy number changes such as a perfectly tetraploidy, will give constant ratios across the genome and be indistinguishable from the result obtained with a diploid cell population. The technique is not capable to detect mosaicism, balanced chromosomal translocations and inversions. Moreover, traditional CGH shows some limitations due to the use of metaphase spreads. First of all, the detection capability is limited to aberrations involving segments of at least 5-10 Mb in size and experimental results is highly influenced by the quality of metaphase spreads 20. Therefore, more laborious locus-by-locus techniques have been required for higher resolution studies. A suitable technique for the study of smaller chromosomal imbalances is FISH, but this approach requires prior knowledge of the target. In the last years, array-based Comparative Genomic Hybridisation (array-CGH), a new method combining the resolution of FISH with the whole-genome screening capacity of array-CGH, as been introduced in molecular cytogenetics 16 17. Thanks to these features, array-CGH has immediately revealed a great potential for the study of chromosomal imbalances in clinical genetics.
Fig. 2 | Comparative Genomic Hybridisation experiment. A) Test and reference DNAs are labelled in SpectrumGreen and SpectrumRed respectively and simultaneously hybridised to a normal metaphase spread. B) Color ratio peak profile of the CGH experiment. The central black graph bar represents a ratio of 1. Areas to the right and to the left represent gains or losses in DNA copy number. The green line indicates the threshold value (1.25) for copy number gains and the red line indicates the threshold value for copy number losses (0.75). \( n \) represents the number of chromosomes analysed (adapted from Floridia G. et al, 2005)
1.2.2 The array-based Comparative Genomic Hybridisation

In the array-based Comparative Genomic Hybridisation technique (array-CGH) metaphase chromosomes spreads are replaced by the use of DNA immobilized on solid supports, representing a significant advantage in terms of quantity accuracy, resolution and repeatability with respect to traditional CGH. A microarray is an analytical device formed by an array of molecules (BAC or PAC clones, cDNAs, oligonucleotides, PCR products, polipeptides etc) or tissue sections immobilized at discrete locations on a solid porous or nonporous support. The distance between each immobilized target may vary from some millimetres to few micrometers depending on the microarray type \(^{21}\). In the context of microarrays, the molecule immobilized on the slide is indicated as probe, while the target is the molecule in solution exposed to the array \(^{21}\). Microarray formats for array CGH have been developed over the last 10 years \(^{16}\). The use of an array of mapped probes instead of metaphase spreads allows to overcome the main limitation of conventional CGH, that is the low resolution. Theoretically, the resolution of an array-CGH slide is limited only by the genomic distance between each DNA probe represented on the array (density of the probe) and by the size of the spotted sequences. However, to calculate the array resolution based on the mean of distances between probes would be misleading. In fact, array elements may be not evenly distributed throughout the genome and some platforms may require multiple probes to detect an alteration \(^{22}\). A functional measure of resolution can be the size limit of detecting a segmental copy number alteration. Basic principles of array-CGH resembles that of conventional CGH. Test and reference DNAs are differentially labelled with green and red fluorochromes, then co-precipitated in presence of Cot-1 DNA to block repetitive sequences and co-hybridised onto an array. The slide is subsequently analysed with an array scanner and images are digitally quantified with dedicated softwares (Fig.3). The ratio of fluorescent intensities for each probe
represented on the slide is normalized and plotted against the genome sequence position. Therefore, array-CGH allows to identify genomic copy number alterations. In addition, given that measurements can be referred directly to the positions on the genome it is possible to directly characterized the breakpoints of the rearrangement. The resolution of the experiment depends on the resolution of the array.

The main advantages in array-CGH technique application compared to conventional cytogenetic and other molecular cytogenetic approaches are: i) dividing cells are not required; ii) the resolution is extremely high (virtually, it is possible to design arrays covering any target chromosomal region with any desired resolution); iii) whole-genome may be analysed in a single experiment (with a screening potentiality equivalent to that of thousand FISH experiments); iv) sensitivity and specificity are very high 23, 24. Although array-CGH has proved to be an efficient and reproducible technique, the structural configuration of the abnormal chromosomes could not be characterized. The order and the orientation of the rearranged segments cannot be determined, it is not possible to distinguish a perfect polyploidy from a normal karyotype and also low levels of mosaicism may be difficult to detect (fig. 4). These limitations depend on the general principles of the methodology, while array-CGH performances may be strongly dependent also from the type of array-CGH platform employed 19.
Fig. 3 | **Schematic representation of an array-CGH experiment.** Test and reference DNA are differentially labelled, co-precipitated and hybridised to an array. After wash procedures, the slides are analysed through a scanner and fluorescence intensities of each probe are determined. After imaging processing and data normalization, the log2 ratios of the probes are plotted as a function of chromosomal position. Probes with a value of zero represent equal fluorescence intensity ratio between sample and reference. Each dot represents a single probe spotted on the array. In this representation, copy number loss shift the ratio to the left and copy number gains shift the ratio to the right.
Fig. 4 | Comparison of standard banding techniques and array-based approaches for identifying chromosomal abnormalities. Various chromosomal aberrations that might be present in clinical samples are shown. A + indicates that the technique is suited for identifying the chromosomal imbalance; a -, indicates that the aberration would be missed. (+)’ indicates that a numerous of small subpopulations has to be analysed to detect the aberration. DM=double minutes; HSR=homogeneously stained region (modified from Speicher MR et al, 2005[1]).
1.2.2.1 Array-CGH platforms

The technical differences between the different array-CGH platforms may be substantially summarized in two features: 1) the size of the genomic sequences spotted on the array and 2) the coverage of the genome. Consequently, arrays platforms are first of all classified on the basis of the type of probes used, as summarized in Table 1.

Array platforms using large insert clones such as cosmid, PAC clones or BAC clones, are precise and accurate and characterized by a wide dynamic range of copy number variations detection, including single copy number gains and losses, homozygous deletions and high-level amplifications. Moreover, the size of the spotted elements guarantees an high signal to noise ratio \(^{17}\). Large insert clones arrays are available both for whole-genome analysis with an average resolution of about 1 Mb and for chromosome specific studies at an higher resolution of about 200-300 kb \(^{25,26}\). Ishkanian et al (2004) developed a full coverage DNA microarray using about 30 000 BAC clones spotted in triplicate on two independent array slides, obtaining an average resolution of approximately 0.1Mb \(^{27}\). However, producing a BAC array with a such wide number of BAC clones is expensive and time-consuming and considering the size of BACs, the resolution limits of BAC – arrays CGH has been reached. The major consideration in interpreting whole-genome BAC array data is the fact that some clones may map to multiple locations in the human genome. The inclusion of these clones on the array is necessary when a tiling path BAC array is constructed, consequently their presence must be computationally tracked to have a correct interpretation of the results \(^{22,28}\).

The first platforms introduced for whole-genome array-CGH screening were cDNA arrays, originally developed for expression profiling and subsequently employed for evaluate copy number variations in breast cancer cell-lines and tumours \(^{29}\). Using this assay it is possible to directly compare copy number variations
and expression data of the same sample. Although highly effective, this method has the limit to cover only expressed regions of the genome, encompassing a maximum of 13824 genes with an average resolution of 267 kb \(^{30}\). More recently, using cDNA probes an exon array-CGH method has been developed \(^{31}\). This assay allows to screen the human genome for copy number changes at the resolution of the individual exons. At present, this method is actually limited to studies focused on specific genomic regions, given that the application of this approach to a whole genome screening requires a great technological improvement.

Oligonucleotide arrays were introduced to detect single-nucleotide polymorphisms (SNP) \(^{32}\). This array type contains 21-25 mer probes synthesized used a photolithographic method. Each SNP is represented on the array by several different probes that interrogate the site both on the sense and the antisense strand\(^{32}\) \(^{33}\). Bignell et al (2004) optimized a method to employ SNP-arrays for copy number variations analysis, using a different strategy for target preparation \(^{32}\). Sample DNA was prepared using whole-genome sample assay (WGSA), a PCR-based method performed to enrich sample DNA for small XbaI restriction sites. This method reduces sample complexity prior to hybridization and decreases the probability of cross-hybridization when small 25-mer probes are used. In SNP-arrays applications the test and the normal samples are hybridized to different arrays. The SNP platform allows the identification of deletions/duplications but shows greater variation in detection capability and a lower signal to noise ratio with respect to BAC arrays \(^{22}\) \(^{32}\) \(^{34}\). The advantage of this approach is the possibility to relate copy-number and allelic status at selected loci. Subsequently, oligonucleotides spotted arrays containing more longer probes (60-70 mer) were developed. The use of typically 60-mer probes increases hybridisation specificity and improves the signal to background ratio to a level that is comparable to that of BAC arrays on a whole-genome scale. In addition, oligonucleotides-libraries are cheaper, easier to work with and faster than cDNAs or BAC/PAC clones, because no DNA isolation or PCR amplification steps are necessary \(^{33}\) \(^{35}\). This array-CGH platform can reproducibly detect genomic lesions, including single copy and homozygous deletions with an extremely high resolution \(^{35}\) \(^{36}\) \(^{37}\) \(^{38}\). Whole genome oligonucleotide arrays with a resolution of about 35 kb are currently commercially available (Table 1).
Table 1. Genomic microarray platforms for whole genome array CGH (* adapted from: Lockwood WW et al, Eur J Hum Genet (2006))

<table>
<thead>
<tr>
<th>Probes</th>
<th>Manufacturer</th>
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<tr>
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<td>Sanger</td>
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<tr>
<td>Large clones</td>
<td>DFKFZ (Deutsches Krebsforschungszentrum)</td>
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<tr>
<td>Large clones</td>
<td>AFCRI (Abrahamson Family Cancer Research Institute)</td>
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<tr>
<td>Large clones</td>
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<tr>
<td>Large clones</td>
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<td>long oligonucleotides</td>
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<td>short oligonucleotides</td>
<td>Affymetrix</td>
<td><a href="http://www.affymetrix.com/">http://www.affymetrix.com/</a></td>
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The flexibility to design genome-wide or chromosome and locus specific custom microarrays and the possibility to cover human genome with an extremely high resolution, makes the array-CGH approach ideal for clinical genetic applications such as identification of disease genes, genotype-phenotype correlation and analysis of cryptic chromosomal anomalies.

1.2.2.1 Applications of array-CGH in clinical genetics

In the past few years, microarray-based format for CGH are beginning to be widely used in preference to chromosome-based CGH for the identification of chromosomal regions that are currently lost or gained in tumours. Both targeted and whole genome arrays have been used to identify specific imbalances in different tumour types.

At present, array CGH methods are increasingly been used for the analysis of patients with complex phenotypes. Due to numerous studies reporting subtelomeric imbalances in patients with developmental disorders, one of the first applications of array CGH was the screening for subtelomeric rearrangements.

Genome wide approaches are of great importance to investigate the presence of submicroscopic deletions or duplications in patients with mental retardation and dysmorphisms. In a study including 90 patients, submicroscopic aberrations were identified in about the 21% of cases. The powerful of this approach may result of particular importance in the identification of specific disease loci in sporadic malformation syndromes, for which other mapping methods are not applicable. Recently, Vissers et al (2004) using this approach discovered that CHARGE syndrome is due to haploinsufficiency of the CHD7 gene demonstrating the efficacy of the array-CGH approach in localize disease-causing genes. Veltman et al (2003), studied 20 patients with congenital aural atresia in order to localize the gene responsible for this malformation with a deletion mapping approach using a high resolution BAC array specific for chromosome 18. They identified a 5 Mb critical
region on 18q22.3-q23. The same approach was tentatively used for other sporadic syndromes, such as Kabuki syndrome.\(^{47}\)

Tiling path arrays are very useful in mapping and sizing of segmental aberrations, allowing accurate genotype-phenotype correlation studies.\(^{48, 49}\) Yu et al (2003) used a target tiling path array to accurately define patients with 1p36 deletion syndrome. This array type containing 10 Mb of the 1p terminal region allowed to correctly classify patients on the basis of their chromosomal alteration (terminal deletion, interstitial deletion or derivative chromosome)\(^{50}\).

Several studies used array CGH to investigate patients with apparently balanced chromosome rearrangements and abnormal phenotype. In these patients the abnormal phenotype may be explained by the disruption of a gene, a positional effect or a cryptic genomic imbalance at the breakpoint or in another region of the genome. Tiling path array-CGH was successfully employed to identify cryptic imbalances at the chromosomal breakpoints.

Finally, array CGH has provided important knowledge about normal variation in the human genome. Using array-CGH the human genome revealed an unexpected level of variation due to copy number differences between individuals.
1.3 Array-CGH and copy number variations in the human genome

It is estimated that about the 5% of the human genome is represented by segmental duplications \(^{51}\). Segmental duplications are defined as stretches of DNA sequence \(\geq 1-5\) kb in length with \(\geq 90\%\) sequence conservation that are present in more than one location in the genome \(^{52}\). Segmental duplications may be composed of genic sequence with introns and exons, as well as common repeats, such as Alu and L1 elements and appear to be “normal DNA”. Duplications may occur in tandem, but they are usually interdispersed. They can be both intra- and interchromosomal duplications, when are present on homologue chromosomes \(^{51}\). Many studies have noted a significant association between the location of segmental duplications and regions of chromosomal instability or evolutionary rearrangement \(^{53}\). The duplication architecture of the human genome may mediate not only recurrent pathogenic rearrangements, but also normal variation \(^{54}\). Variation in the human genome is present in many forms, including single-nucleotide polymorphisms, small insertion-deletion polymorphisms, variable numbers of repetitive sequences and genomic structural alterations, that are large duplications and deletions \(^{55}\). In particular, differences in gene copy number could be a significant source of genetic variation between humans \(^{56}\). Molecular genetics and cytogenetics studies allowed to identify several variations in the human genome. However, only recently it has been possible to study submicroscopic copy number variations (CNVs). Usually these copy number changes are comprised between 1 kb and 3Mb in length. The frequency of CNVs in the human genome and which is their contribution to human diversity is presently unknown. New developments in genome-wide scanning techniques for copy number variations highlight variation in normal population, providing the opportunity to generate advanced maps of structural variation in worldwide population \(^{57-59}\). If the frequency of the variation is \(>1\%\), it is called copy number polymorphism (CNP). Some authors studied the
frequency of CNPs in normal individuals 55 56 60. These authors used different microarray-based approaches and reached substantially concordant results about the presence of several CNPs in normal individual that often involve known genes, also suggesting an enrichment of segmental duplications in the neighbour of duplicated/deleted regions. Sharp et al (2005) used a “segmental duplication microarray” to investigate copy number variations in control individuals of different ethnicity 53. This targeted BAC array covered 130 regions of potential genomic instability named “rearrangement hotspots” and defined by the presence of intrachromosomal segmental duplications >10 kb and with an high grade of homology (>95%). The use of this targeted approach allowed to confirm previously reported data and to improve the ascertainment of structural rearrangements, confirming that genomic duplication architecture is strongly associated with CNPs in human genome.

CNPs may have a role both in the genetics of complex traits and in genome evolution 61. The presence of a number CNPs that are present almost without exceptions in multiple populations, assume great importance when array-CGH is translated in diagnostic settings. The potential difficulties in differentiate between inherited copy number variations which cause abnormal phenotypes and rare variants unrelated to clinical alterations currently may represent a limitation in the use of CGH – microarray for guiding genetic counselling. Therefore, it is necessary to improve our knowledge about the type and the frequency of normal variations in human genome. Several database containing information on genomic variability in normal individuals are available on line (Table 2).
Table 2. Examples of databases for structural variations in the human genome

<table>
<thead>
<tr>
<th>Database</th>
<th>Description</th>
<th>Web address</th>
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<tbody>
<tr>
<td>Database of Genomic Variants</td>
<td>Human large scale genomic variants with information about frequency and map elements</td>
<td><a href="http://projects.tcag.ca/variation">http://projects.tcag.ca/variation</a></td>
</tr>
<tr>
<td>DECIPHER</td>
<td>Sub-microscopic chromosomal imbalances and information on the resulting phenotype</td>
<td><a href="http://www.sanger.ac.uk/genomes/decipher">http://www.sanger.ac.uk/genomes/decipher</a></td>
</tr>
<tr>
<td>Developmental Genome Anatomy Project</td>
<td>Balanced chromosomal rearrangements critical to development</td>
<td><a href="http://www.bwhpathology.org/dgap">http://www.bwhpathology.org/dgap</a></td>
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<td>European Cytogeneticists Association</td>
<td>A collection of cytogenetic, molecular and clinical data of rare unbalanced chromosomal aberrations</td>
<td><a href="http://www.ecruruca.net">http://www.ecruruca.net</a></td>
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1.4 Submicroscopic chromosomal rearrangements in patients with mental retardation and complex phenotypes

Since karyotyping became a routine technique in clinical genetics, mental retardation with or without other abnormalities has often been found to be associated with chromosome rearrangements. However, in the majority of the patients the conventional cytogenetic analysis gives a normal result and in about the 50% of the cases no obvious cause for the impairment is found. It has been demonstrated that up to 5-7% of patients with mental retardation and dysmorphisms presents submicroscopic chromosome imbalances. Array-based genome wide copy number screening is expected to have a profound impact on the diagnosis and genetic counselling of patients with congenital mental retardation and malformation. Vissers (2003) and Shaw-Smith (2004) demonstrated that about the 25% of patients with mental retardation and dysmorphisms and apparently normal karyotype carried deletions or duplications below the level of resolution of classical cytogenetics. More recently, Rosenberg et al (2006) reported the analysis of 81 patients with mental impairment, facial morphologic anomalies and congenital abnormalities with array-CGH (1 Mb resolution). All the patients had a normal G-banded conventional karyotype and not ascertained cause of disease. Authors identified chromosomal imbalances in the 25% of cases, confirming previously reported data.
2. RATIONALE, AIM and OUTLINE
2. RATIONALE, AIM and OUTLINE OF THE STUDY

Chromosomal abnormalities are a major cause of mental retardation and congenital malformations. It is known that a considerable fraction of patients with multiple congenital anomalies and mental retardation have submicroscopic chromosomal imbalances. The introduction of whole genome array-CGH allows to investigate the DNA for the presence of copy number alterations with high resolution. In patients with multiple congenital anomalies and mental retardation 15-24% of segmental aneusomies were reported. Given these data, we have decided to apply both classical approaches and innovative methodologies to the study of several patients with mental retardation and congenital anomalies.

First, we have worked on the set up of the array-CGH protocols. We employed two different types of commercially available array platforms, a BAC array slide with a resolution of 1 Mb and an oligonucleotide array with a resolution of 75 kb (Result 1).

After the set up of the methodology, we selected a first group of patients with mental retardation and multiple congenital anomalies for array-CGH investigation. Patients available for the study are inserted in a biobank coordinated by the Medical Genetics of the University of Siena and are inserted in a on-line database (http://xlmr.unisi.it). The database contains cases of familial and sporadic mental retardation with or without other anomalies that are collected through the activity of the X-linked Mental Retardation Italian Network, active since 2003. The XLMR bank is an innovative biological database that allows the collection of molecular and clinical data that combines descriptive and iconographic resources and represents a fundamental tool for researchers in the field of mental retardation (Result 2).

We started the study of patients with complex phenotypes working on the molecular characterization of a female patient with a complex phenotype,
resembling some clinical features typical of Rett syndrome. The patient presents a de novo deletion of chromosome 2q initially identified with a standard G-banded karyotype. We used a classical approach with the employ of STR markers to better define the deletion breakpoints and we performed an in silico analysis of the gene content of the deleted region in order to select gene potentially involved in generating the phenotype for further studies (Result 3).

Given the availability of new technologies to study copy number variations in the human genome, we decided to set up array-CGH technique in our laboratory to investigate patients with mental retardation and multiple congenital anomalies and normal G-banded karyotype for the presence of submicroscopic imbalances.

At present, we have analysed by array-CGH 15 patients with mental retardation, facial morphologic anomalies and congenital anomalies. We have identified four de novo chromosomal aberration and one inherited deletion. In particular, we have identified a 2q interstitial deletion of about 10 Mb in a female patient with developmental delay, severe seizures and dysmorphic features (Result 4). In addition, we have characterised two patients with 13q deletion syndrome and we have studied another case with retinoblastoma and developmental anomalies that shows only a maternally inherited 7q deletion (Result 5).

Finally, we have found an additional 2q non overlapping deletion in a case of severe mental retardation and behavioural problems (Result 6).

This study allowed the characterization of several chromosomal imbalances in patients with complex phenotype, confirming the power of the array-CGH method to clarify the molecular basis of these difficult cases. Through the employ of this innovative approach, several families finally received a definitive diagnosis and a correct recurrence risk assessment.
3. MATERIALS and METHODS
3. MATERIALS & METHODS

3.1 Patients collection

Patients with mental retardation and multiple congenital anomalies involved in the study described here are been collected through the activity of the XLMR Italian Network. The XLMR Italian Network involves several clinical centres and laboratories spread all-over Italy with the aim of collect a vast number of patient with mental retardation and with/without other anomalies. At present, 27 clinical centres have agreed the proposal recruiting 140 patients with mental retardation. The collected patients are registered in a web-based database available at the web-address <http://xlmr.unisi.it>. The database is conceived to stored molecular data and detailed clinical information both on the patients and their families. The biological samples (DNA, lymphoblastoid cell lines, lymphocytes in DMSO medium) of the patients and their familiars are mainly stored at the Medical Genetics Unit of the University of Siena, that acts as coordinator of the project. The twelve laboratories that have joint the Network are engaged in performing molecular analysis of some mental retardation genes, array-CGH experiments, X-Inactivation studies and enzymatic assays on the collected samples.

For an accurate description of the activities of the Network and of the working of the electronic database see Result 1.
3.2 Array-based CGH

3.2.1 Samples preparation

Genomic DNA of normal controls was obtained from Promega. Genomic DNA was extracted from peripheral blood using a QIAamp DNA Blood Maxi kit according to the manufacturer protocol (Qiagen, www.qiagen.com). The OD260/280 method on a photometer was employed to determine the appropriate DNA concentration \(^{68}\). Patient and control DNA samples were sonicated to produce a homogeneous smear DNA extending from approximately 600 bp to 2 kb. DNA samples were then purified using the DNA Clean and Concentrator kit (Zymo Research, Orange, CA). Ten micrograms of genomic DNA both from the patient and from the control were sonicated. Test and reference DNA samples were subsequently purify using dedicated columns (DNA Clean and Concentrator, Zymo research, CA92867-4619, USA) and the appropriate DNA concentrations were determine by a DyNA Quant™ 200 Fluorometer (GE Healthcare).

3.2.2 Human BAC arrays

Array-based comparative genomic Hybridisation was performed using commercially available whole-genome BAC arrays. The Spectral Genomics DNA array slides (Human BAC Array, Spectral Genomics, Houston, TX) contains 2600 BAC clones spotted in duplicate with a resolution of 1 Mb. The IntegraChip arrays (Integragen – Evry France) contains 3200 BAC clones spotted in quadruplicate with a resolution of 0.8 Mb. Labelling and hybridisation were performed according to the manufacturer’s protocol. Both forward and dye swap labelling experiments were done for the patient.
Following hybridisation, Spectral Genomic slides were scanned on a GENEPIX 4000B scanner (Axon Instruments, Union City, CA) and the TIFF images captured using GENEPIX Pro Software. The images were analysed using SPECTRALWARE BAC Array Analysis Software v 2.0 (Spectral Genomics). The software recognizes the regions of fluorescent signal, determines signal intensity and compiles the data into a spreadsheet that links the fluorescent signal of every clone on the array to the clone name, its duplicate position on the array and its position in the genome. The software was also use to normalize the Cy5:Cy3 intensity ratios for each slide and each data point. The normalized Cy5:Cy3 intensity ratios were computed for each of the two slides and plotted together for each chromosome. The ratio plot is arbitrarily assigned such that gains in DNA copy number at a particular locus are observed as the simultaneous deviation of the ratio plot from a modal value of 1.0, with the blue ratio plot showing a positive deviation while the red ratio plot shows a negative deviation at the same locus. DNA copy number losses show the opposite pattern. The linear order of the clones is reconstituted in the ratio plots consistent with an ideogram, such that the p terminus is to the left and the q terminus is to the right of the plot.

After hybridisation and washing procedures, IntegraChip arrays were scanned on a ScanArray Gx scanner (PerkinElmer Spa Italia; http://las.perkinelmer.it). Spot analysis and quality control were fully automated using BlueFuse version 3.1 (BlueGnome, Cambridge, UK).

3.2.3 Human oligonucleotides array

Array based CGH analysis was performed using commercially available oligonucleotide microarrays containing about 43,000 60-mer probes with an estimated average resolution of 75 kb (Human Genome CGH Microarray 44B Kit, Agilent Technologies). DNA labelling was executed essentially according to the Agilent protocol (Oligonucleotide Array-Based CGH for Genomic DNA Analysis
2.0v) using the Bioprime DNA labelling system (Invitrogen). Genomic DNA (2 μg) was mixed with 20 μl of 2.5X Random primer solution (Invitrogen) and MilliQ water to a total volume of 41 μl. The mix was denatured at 95°C for 7 minutes and then incubated in ice/water for 5 minutes. Each sample was added with 5 μl of 10X dUTP nucleotide mix (1.2 mM dATP, dGTP, dCTP, 0.6 mM dTTP in 10 mM Tris pH 8 and 1 mM EDTA), 2.5 μl of Cy5-dUTP (test sample) or 2.5 μl of Cy3-dUTP (reference sample) and with 1.5 μl of Exo-Klenow (40 U/μl, Invitrogen). Labeled samples were subsequently purified using CyScribe GFX Purification kit (Amersham Biosciences) according to the manufacturer protocol. Test and reference DNA were pooled and mixed with 50 μg of Human Cot I DNA (Invitrogen), 50 μl of Blocking buffer (Agilent Technologies) and 250 μl of Hybridization buffer (Agilent Technologies). Before hybridization to the array the mix was denatured at 95°C for 7 minutes and then pre-associated at 37°C for 30 minutes. Probes was applied to the slide using an Agilent microarray hybridization station. Hybridization was carried out for 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 with the Agilent 44B kit. The slides was dried and scanned using an Agilent G2565BA DNA microarray scanner. Image analysis was performed using the CGH Analytics software v. 3.1 with default settings. 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 linear order of the oligos is reconstituted in the ratio plots consistent with an ideogram. The ratio plot is arbitrarily assigned such that gains and losses in DNA copy number at a particular locus are observed as a deviation of the ratio plot from a modal value of 1.0.
3.3 Real-time quantitative PCR

Array – CGH data were confirmed by Real-time Quantitative PCR experiments. To design adequate probes in different regions of the human genome, we used an TaqMan Gene Expression Assays by design which provides pre-designed primers-probe set for real-time PCR experiments (Applied Biosystems, https://products.appliedbiosystems.com; for probes description see Results 3 and 4). PCR was carried out using an ABI prism 7000 (Applied Biosystems) in a 96-well optical plate with a final reaction volume of 50 µl. A total of 100 ng (10 µl) was dispensed in each of the four sample wells for quadruplicate reactions. Thermal cycling conditions included a prerun of 2 min at 50°C and 10 min at 95°C. Cycle conditions were 40 cycles at 95°C for 15 sec and 60°C for 1 min according to the TaqMan Universal PCR Protocol (ABI). The TaqMan Universal PCR Master Mix and Microamp reaction tubes were supplied by Applied Biosystems. The starting copy number of the unknown samples was determined using the comparative Ct method as previously described ⁶⁹
4. RESULTS
4.1 Evaluation of performances of 1 Mb resolution BAC arrays and tiling path oligonucleotide-arrays

*Unpublished results*
Evaluation of performances of 1Mb resolution BAC arrays and tiling path oligonucleotide arrays.

In order to evaluate the performances of two distinct commercially available platforms, we have analyzed a patient with a 5 Mb 15q deletion using both 1 Mb BAC arrays and tiling path oligonucleotide arrays (average resolution of about 75 kb). Using the BAC array, the 5 Mb deletion was pointed out by a single clone with a ratio value of about –0.5 (Fig. 5, left panel). The analysis by oligonucleotide array-CGH revealed the presence of a chromosome 15 interstitial deletion extending from a minimum of 4.91 Mb to a maximum of 5.75 Mb (46,XY del(15)(q11-q13.1)) (Fig.5, right panel). The proximal breakpoint is mapped in 15q11 (last oligonucleotide present located in 21.25 Mb, first deleted in 21.29 Mb position), while the distal breakpoint is located between 26.19 Mb and 26.99 Mb in 15q13.1 (last oligonucleotide deleted and first present, respectively).
Fig. 5 | **Array-CGH characterization of the 15 q deletion.** On the left, the result of the array experiment performed using the 1 Mb whole BAC array analysed with the BlueFuse software. Note that the 5 Mb deletion is pointed out by a single clone showing a ratio value of about −0.5. On the right, the result of the array experiment with the employ of an oligonucleotide tiling path array.
4.2 The Italian XLMR bank: a clinical and molecular database.


*submitted to* Human Mutation, April 2006
The Italian XLMR bank: a clinical and molecular database.


1 Medical Genetics, Department of Molecular Biology, University of Siena, Italy

ABSTRACT

Mental retardation is a non-progressive condition characterized by a significant impairment of intellectual capabilities with deficit of cognitive and adaptative functioning and onset before 18 years. Mental retardation occurs in about 2-3% of the general population and it is estimated that the 25%-35% of cases may be due to genetic causes. Given the genetic heterogeneity of XLMR, the availability of a considerable number of patients with accurate phenotypic classification is a crucial factor for MR research. The X-linked Mental Retardation Italian Network is active since 2003 to collect detailed clinical information and biological samples from a vast number of patients with mental retardation. Collected samples and clinical information are inserted
within the XLMR bank. The XLMR bank is a comprehensive molecular and clinical web-based database available at the address http://xlmr.unisi.it. The database is organized in three distinct parts. Part I and II of the database contain several electronic schedules to register information on the family and the phenotypic description, the photographs and a 20 sec movie of the patient. Part III allows the registration of molecular analysis performed on each case inserted in the bank. Samples and clinical data are usable through a password restricted access. Clinical and molecular centres interested to joint the Network may request a password simply contacting the Medical Genetics of the University of Siena. The XLMR bank is an innovative biological database that allows the collection of molecular and clinical data, that combines descriptive and iconographic resources and represents a fundamental tool for researchers in the field of mental retardation.

Key words: XLMR, X-linked mental retardation, molecular database, clinical database, XLMR Italian Network.

INTRODUCTION

Mental retardation (MR) is a non-progressive condition characterized by a significant impairment of intellectual capabilities with deficit of cognitive and adaptative functioning and onset before 18 years [Chelly and Mandel, 2001]. General intellectual functioning is defined by the “intelligence quotient” (IQ) and adaptative functioning refers to how individuals deal with common life demands. Mental retardation occurs in about 2-3% of the general population [Gecz, 2004] and represents an important socio-economic and medical issue, given that MR patients need a continuous support from families and health-care operators. It is estimated that a percentage of MR cases between the 25% and the 35% may be due to genetic causes. On the basis of the IQ value, mental retardation may be classified in four categories of severity: mild (IQ 50-70), moderate (IQ 35-50), severe (IQ 20-35) and profound (IQ >20). Moreover, mental retardation may be present in association with other clinical manifestation (syndromic mental retardation) or may be isolated (non-syndromic mental retardation) [Mulley, et al., 1992]. Males are more frequently affected than females, with an excess of about 30% of male cases explained by the presence of several MR causative genes on the X chromosome responsible for the X-linked cases of MR (XLMR) [McLaren J, 1987] [Baird and Sadovnick, 1985] [Neri and Chiurazzi, 1999]. At present, 27 genes for non-syndromic XLMR and 45 genes for syndromic XLMR are known [Renieri et al., 2005] [Bauters M et al., 2005]. Some genes are responsible of both syndromic and non-syndromic forms of XLMR and most of them are responsible for a very low percentage of XLMR cases, comprised between the 0.1 and the 1%. The abundance of known XLMR genes and the fact that no major genes exists makes difficult to offer a molecular diagnosis to MR patients. Given the genetic heterogeneity of XLMR, the availability of a considerable number of patients with accurate phenotypic classification is a crucial factor for MR research. The X-linked Mental Retardation Italian Network includes 12 laboratories and 27 clinical centres and is active since 2003 to collect a vast number of MR patients with detailed clinical information according to common criteria, and to analyse known
MR genes. It collects both sporadic and familial mentally retarded male patients negative for FRAXA, chromosomal and subtelomeric rearrangements. Biological samples are conserved in a biobank located in the Medical Genetics laboratory of the University of Siena and in the Genetic Institute of the Università Cattolica del Sacro Cuore in Rome. Collected samples and the clinical information are inserted in a web-based database available at the address http://xlmr.unisi.it through a password restricted access. All centres interested to join the Network can contact the Medical Genetics of the University of Siena.

We describe here the “XLMR bank” a comprehensive web-based database that includes accurate clinical and molecular data on the MR patients present in the biobank. Samples and clinical data are available on request for specific studies. The “XLMR bank” represent an important tool for the researchers involved in discover the molecular genetic bases of autosomal and X-linked MR and clarify the pathogenic processes that underlain the disease.

THE XLMR BANK

The “XLMR bank” is available at the web address http://xlmr.unisi.it and it is maintained and updated on the server of the University of Siena. The database is created using the Access software (Microsoft Office; http://www.microsoft.com) for data management. The web-site is written in VBScript and takes advantage of a Microsoft IIS (Internet Information Server) server with ASP (Active Server Pages) technology.

Database structure
The home page of the “XLMR bank” (http://xlmr.unisi.it/homepage.asp) contains general information about the project. In particular, it explains the aims and the overall organization of the project (clinical centres and laboratories involved, patients enrolled and analysed genes). Using the “Search” option the external users can visualize the complete list of patients collected in the database and visualize each pedigree and the provisional or definitive diagnosis (Fig. 1).
Figure 1. Home page of the XLMR bank ([http://xlmr.unisi.it](http://xlmr.unisi.it)). Using the “Search” option available at the home page, users can visualize the complete list of patients recorded in the bank and consult their pedigree. This set of information is available also for the “external users” without a password access.

The overall design of the “XLMR bank” database includes three distinct parts with a password restricted access (Fig.2). Each part is organized in several electronic schedules, in order to collect detailed clinical and molecular data. Part I of the database includes forms filled by the clinical centre that have visited the patient and collects the biological samples (Fig.3).
Figure 2. Overall design of the “XLMR bank” on-line database. The database includes three distinct parts. The access to each part is restricted by passwords. The levels of admittance are five, a public level and four different “participants” levels accessible only to centres belonging to the “X-linked Mental Retardation Italian Network”. Symbols: ⬛ indicates information not accessible to the users; ⚠ shows the schedules that may be filled and/or modified with the access of the indicated level; ⏯ indicates the information that are visible but not modifiable. The information on the diagnostic hypothesis is available for users of all levels. Anagaphical data are visible only for the bank curator who has a fifth level access.

The forms are divided in the following specific sections: 1) Private data; 2) Family History (pedigree, clinical data and photographs of the relatives); 3) Anamnestic Form (clinical history of the patient, IQ value, adaptative functioning evaluation and behavioural tests, laboratory results for standard cytogenetic analysis, subtelomeric rearrangements investigation, fragile-X test); 4) Clinical data of the patient (clinical features, morphologic examination, neurological data, JPEG format photographs and 20’’ movie whenever relevant); 5) Privacy (name and address of the physician that retains the informed consent); 6) Diagnosis (provisional diagnostic hypothesis); 7) Trait description, using conventional dysmorphology dictionary (from LDDB and Possum databases); 8) Shipped biological samples (collected samples and biobank address). To guarantee confidentiality, each sample in the database is univocally identified by a code of three numbers separated by an hyphen: a progressive number generated by the system that characterized the family and it is the same for each family member and two numbers indicating the pedigree position of the subject (Fig.3I).
Figure 3. Part I of the “XLMR bank” database. The access to this section is password restricted (panel A). Each panel indicates the different schedules included in this section and filled by the clinical centre that have visited the patient (panels from B to I).

Part II is filled by the biobank curators and includes information on the source and the type of the stored biological samples (Fig. 4). Currently, the following biological samples are stored: DNA, plasma, DMSO stored lymphocytes and lymphoblastoid cell lines. Part III includes information about the laboratory tests performed on each collected samples (Fig. 5). This part is filled by the laboratories that join the Network and execute the molecular analysis. The laboratory tests currently executed are listed in Table 1.
Figure 4. Part II of the “XLMR bank” database. This section of the database includes information on the source and the type of the stored biological samples. It is possible to add new samples simply clicking on the check box corresponding to the correct sample type stored (i.e. DNA sample). Note that the code is generated by the system when a new patient is registered in the bank. The same code is attributed to each sample obtained from that patient.

Database accessibility and clinical and molecular data management

The database is organized on five levels of admittance, a public level and four different “participants” levels, accessible only through a password, to centres belonging to the “X-linked Mental Retardation Italian Network” (Fig.2). The first level is freely available to the external users going to the “Search” option. External users can freely visualize the pedigree of each case. Specific information is accessible through a Login page only for participants and it includes detailed clinical data, location and preservation of biological samples (DNA, lymphoblastoid cell line, plasma, urine samples etc.), molecular data. Second level password is assigned to clinical centres which visit the patient and decide to insert the case in the XLMR bank. Clinical centres collect the biological samples and send them to the laboratories qualified to the storage. A laboratory qualified to the storage has to guarantee the establishment and maintenance of lymphoblastoid cell lines. Using a second level password, the clinical centre can see and modify clinical data limited to patients they have inserted and only consult their molecular data.
A third level password allows to the clinical centres to visualize clinical and molecular data of all the patients in the bank. Laboratories that perform patients analysis have a fourth level password and allow to fill Part III of the database. Each laboratory can visualize molecular information inserted by other laboratories. The fifth level password is the administrator level. Bank curators only have this access that allow to visualize and modify all the information maintained in the database, including anagraphical data.

**Security and quality assurance**

The XLMR bank is available to all users who contact the bank curators and fill in and sign a specific form to join the Network on Mental Retardation and to receive a new password. The Network members agree the rules of the bank and commit themselves to not distribute the password and the information maintained in the database.

To prevent the accidental damage of samples, storage of biological materials is organized in different –20°C or –135°C freezers. In particular, two aliquots containing at least 400 µg for each DNA samples are stored in two different –20°C freezers and five distinct aliquots are frozen for each lymphoblastoid cell line (four aliquots in the-135°C freezer and one in the liquid nitrogen deware).

**Table 1. Laboratory tests currently executed by the XLMR Italian Network members.**

<table>
<thead>
<tr>
<th>Mutation Analysis</th>
<th>Gene Name</th>
<th>Laboratory</th>
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Figure 5. Part III of the “XLMR bank” database. Each panel of this section includes information about the laboratory tests performed on each collected samples. Note that different tests may be added or deleted on the bases of the ongoing work.
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<tr>
<td></td>
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<td>Milano</td>
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**Database functions**

Users that visit the on line database can search a patient simply looking through the list of all the registered cases. Alternatively, the database content may be explored using the “Search” option available after the login. The search page allows to browse the database selecting for any field the user wants to search for (Fig. 6). It is possible to search by a single field or combining two or more fields. As result of the search, the user will visualize the
list of patients that show the selected feature/s. Information on the selected patients will be available according to the level of the login password.

**Figure 6.** The “Search by” option. This function is available after the login and allows to browse the database selecting for a single field or combining two or more fields. The user will visualize the list of patients that show the selected feature/s. Information on the selected patients will be available according to the level of the login password.

**Discussion**

The primary focus of the XLMR bank is to collect a wide number of both syndromic (unknown syndromes) and unspecific mental retardation cases. Familial cases with an X-linked pattern of transmission are preferred. However, the bank collects also sporadic cases in which the proband is a male. After the first two years, 56 familial cases, 9 likely familial and 81 sporadic cases have been inserted in the database.

An innovative feature of this biological database is the accuracy of the clinical data collection using a combination of descriptive and iconographic tools: text areas, JPEG format photographs and 20” movies. The availability of a detailed description will allow a better definition of the phenotype in that patients in which the molecular defect will be identified and may help researchers in the characterization and definition of new syndromes.

Moreover, a characteristic of the biobank described here is the absence of a main centre storage of biological
samples. All participating laboratories able to establish lymphoblastoid cell lines may propose to store some of the samples. After the first two years of activity, approximately the 70% of biological samples are stored in Siena and 30% in Rome. The sharing of storage activities permits a better use of resources due to a distribution of laboratory costs and to a reduction in postal charges for samples shipments.

The XLMR bank is an ongoing dynamic database. New clinical centres and laboratories can joint the Network at any time just requiring an appropriate level password to the bank curator. This flexibility allows to meet the demands deriving from scientific knowledge. Each newly identified mental retardation gene could be added to the list of the molecular tests performed, as soon as a laboratory will become available. At the same time, the members of the Network may decide to stop the analysis of such genes for which the testing is of unproven utility according to the scientific community.

A clinical centre may ask the appropriate level password just to include a single family or a single patient in the database. This allows a more comprehensive collection of interesting cases that have been visited in small clinical centres spread overall Italy.

To our knowledge the XLMR bank is a unique resource that combines molecular data with accurate clinical information. The XLMR bank with the biobank service and the collection of detailed clinical information represents an important tool for researchers in the field of mental retardation. The sharing of the XLMR bank content with the scientific community may give impulse to the identification of new mental retardation causative genes and to the delineation of new syndromes.

Acknowledgments
The XLMR bank is supported by grants from Pierfranco e Luisa Mariani Foundation and from Telethon Foundation (GGP02006; GGP05005) to A.R.

References
4.3 Chromosome 2 deletion encompassing the MAP2 gene in a patient with autism and Rett-like features.


Short Report

Chromosome 2 deletion encompassing the MAP2 gene in a patient with autism and Rett-like features


We present here a unique case of a 14-year-old female with autism and some features similar to Rett syndrome (RTT). Genetic analysis demonstrated a large deletion of chromosome 2q instead of a MECP2 mutation. Like a Rett patient, she is dyspraxic and shows frequent hand-washing stereotypic activities, hyperpnea, and bruxism. Like a preserved speech variant (PSV) of RTT, she is obese, able to speak in second and third persons, frequently echolalic, and has final normal head circumference and autistic behavior. In addition, she has dysmorphic features such as down-sloping palpebral fissures, low set ears without lobuli, bilateral flat feet, and bilateral syndactyly of the second and third toes, which do not belong to the Rett spectrum. She has a de novo chromosomal deletion in 2q34 of paternal origin. Gene content analysis of the deleted region showed the presence of 47 genes (14 putative and 33 known genes). This region contains some interesting genes such as ADAM23, MDC3, CREB1, KLF7, and MAP2. Because alteration of neuronal maturation, dendritic anomalies, and a decrease in MAP2 immunoreactivity in white matter neurons are well documented in RTT patients, we propose the MAP2 gene as a good candidate for the generation of PSV phenotype in this case.

Rett syndrome (RTT) (OMIM 312750) is a severe neurological disorder affecting almost exclusively girls. Classical RTT patients show regression of speech and purposeful hand movements, after a period of normal development. Among the typical clinical features of this condition are hand dyspraxia, stereotypic hand-washing activities, ataxia, abnormal breathing, and growth retardation. About 80% of classical RTT are due to mutations in MECP2 gene, encoding the methyl-CpG-binding protein 2. We also describe some patients affected by the preserved speech variant (PSV) of the RTT syndrome, who are characterized by recovery of the capability to speak. In these less severe cases, autistic behavior is more evident (1–4). Mutations in MECP2 gene are identified in the 50% of PSV cases.

Autism (OMIM 209850) is a defect in the communication, social interaction, and behavior areas. The causes are unknown, but there are strong evidences in favor of a genetic etiology. Recently, a region in 2q33 has been shown to be involved in autism (5, 6). A two-stage genomic screen analysis of autistic patients revealed suggestive evidence for linkage to markers D2S116 (LOD score 2.86) and D2S1384 (LOD score 0.80) (5). These linkage data focus the attention on the presence of a putative autism-causative gene on chromosome 2 and support the hypothesis that behavioral overlapping features of RTT and autism may have common molecular bases.

We present here a Rett-like patient with an intact MECP2 gene and an interstitial deletion in chromosome 2 (2q34) of paternal origin. She has phenotypic features similar to PSV and showed additional dysmorphic signs and an autistic behavior. Analysis of the gene content of the...
deleted region allowed us to identify the presence of gene(s) with a role in neurobiological function or development, which could be a good candidate for the generation of the Rett-like phenotype in the patient.

**Clinical report**

The proband, a 14-year-old girl, was the second child of second cousins once removed consanguineous parents (Fig. 1). The father was 35 years old and the mother, gravida 4, para 1, 31 years old. Pregnancy lasted 36 weeks. Her birth weight was 1920 g and head circumference was 31 cm. Morphologic and neurological abnormalities were noticed at birth: down-slanting palpebral fissures, low set ears without lobuli, syndactyly of the second and third toes in both feet, and simian creases. Her sucking was poor and she was hypotonic. In the subsequent months, her psychomotor development was slow and her ability to relate was poor. An electroencephalogram (EEG) showed no abnormalities. At 9 months, a subluxation of the hip was noticed and was treated accordingly with an orthopedic device that was kept for 4 months. At 18 months, she started a frequent clapping of both hands, which persisted in the following years. At 2 years, she was able to walk alone. At this age, her head circumference was below the norm (45 cm, less than the third percentile), she remained isolated from other people’s interactions, and she started to have frequent bouts of hyperpnea. A computerized tomography of the brain showed enlarged cisterna magna and peripontine spaces, which was confirmed by a subsequent magnetic resonance imaging. An EEG showed frequent spikes, occasionally, followed by slow waves in short sequences on both temporo-occipital leads. At 3 years, grand mal convulsions occurred and remained, in spite of a pharmacological treatment, relatively frequent up to 6 years. Afterwards, they remained well controlled with phenobarbital. At this age, lumbar lordosis, ataxic gait, and hand apraxia were noticed in a department of child neurology, and she had started to say numerous words and short phrases and had just attained sphincter control. She was initially seen by one of us (M.Z.) at 10 years. She had down-slanting palpebral fissures, low set ears without lobuli, bilateral flat feet, and bilateral syndactyly of the second and third toe. Head circumference (51.3 cm) was within the norm (25th percentile), her weight was 45 kg (90th percentile), and she was 149 cm tall (90–97th percentile). She was able to speak in second or third person and was frequently echolalic with an idiosyncratic, out of context speech, characterized by a special prosody, and she fulfilled all the criteria of autism at a DSM IV R evaluation [(1) Full positive, (2) Full positive, (3) Full positive]. She liked music and frequently sang popular songs. She was unable to use a spoon and was evidently dyspraxic. She, however, could build a tower of 10 cubes. In addition, frequent hand-washing stereotypic activities, hand clapping, and cold extremities were noticed. She would walk with an enlarged base and was unable to run. Bruxism was present, and her behavior was occasionally aggressive.

**Cytogenetic and molecular genetic analysis**

Analysis of MECP2 gene was performed by direct sequencing of the coding region of the gene, using PE Big dye terminator cycle sequencing kit on an ABI 310 Automated Sequencer (Applied Biosystems, Foster City, CA, USA) and analyzed with the GENESCAN package software. Primers were described elsewhere (1).

Cytogenetic analysis was performed using standard techniques (7). Chromosomes were prepared from peripheral blood lymphocyte, treated with trypsin, and then stained with Giemsa stain to obtain a GTG-banded pattern.

Polymorphic markers located in the deleted region were identified using both the public database UCSC Genome Browser (http://genome.ucsc.edu) and the private Celera database (http://www.celera.com). Primer sequences are available at The Genome Database (http://www.gdb.org/gdb/). For marker analysis, a precast gradient polyacrylamide gel (12.5–2%) using

*Fig. 1.* Picture of the patient at age of 14 years.
the GenePhor electrophoresis unit (Amersham – Pharmacia – Biotech, Uppsala, Sweden) under a controlled temperature of 10°C was used.

The gene content analysis was performed using public and private databases (UCSC Genome Browser, http://genome.ucsc.edu; Celera database, http://www.celera.com).

Results

Analysis of MECP2 gene showed the presence of a silent variant (p.G273G, c.819G>T) inherited from the father. No additional changes were found, in spite of direct sequencing of the entire coding region.

Cytogenetic analysis using standard techniques showed the presence of an interstitial deletion of chromosome 2. In particular, the karyotype of the patient was 46,XX.del(2)(q34) (data not shown). Both the parents have a normal karyotype.

In order to define the extension and the origin of the deletion, we analyzed a series of polymorphic markers from 2q33.3 to 2q35. This analysis located the proximal breakpoint in a region of about 900 kb between AFM224zg5 (present) and D2S155 (deleted), and the distal breakpoint in a region of about 900 kb between D2S137 (deleted) and D2S301 (present) (Fig. 2). The deletion has an extension of about 9–11 Mb. Analysis of deleted markers showed the absence of paternal contribution, indicating that the deletion arose de novo in the paternal gamete (Fig. 2).

Analysis of the gene content of the deleted region showed the presence of 47 genes (Fig. 3). Of these, 16 are putative genes, while 31 are known genes.

Discussion

We present here a unique case of a 14-year-old female who shows autism and some features similar to RTT syndrome. Genetic analysis demonstrated a large deletion of chromosome 2q instead of a MECP2 mutation. It is interesting to note that the deletion is very near to a region recently confirmed to be involved in autism (5, 6). D2S155, the first deleted marker in our patient, is located 5 Mb telomeric to marker D2S116 which has the highest LOD score (2.86) and 1.7 Mb telomeric to marker D2S1384, which is still included in the linked region (LOD score of 0.80). We cannot exclude that one of the deleted genes may be responsible for the high LOD score in the paper of Shao et al. (6) and may be responsible for autistic behavioral phenotype in the patient.

Direct sequencing of MECP2 has not shown the presence of a causative mutation in this gene. Although MECP2 intronic mutations may be missed and a mutated unknown recessive gene may be present in the patient, de novo origin of the deletion suggests the casual relationship between the deletion and the phenotype in the patient. The identification of this deletion on chromosome 2 opens the challenge of how to assign single phenotypic features to single genes present in the region. Gene content analysis of the deleted region showed the presence of 47 genes.
Thirty-three are known genes and 14 are putative genes. Some of them are of particular interest, due to their role in central nervous system development or to their preferential expression in brain. Some candidate genes with specific neurobiological functions are discussed below.

ADAM23 (a disintegrin and metalloproteinase)/MDC3 (metalloproteinase, disintegrin, and cysteine-rich domain) interacts in brain with a specific integrin (αvβ3) and may mediate cell-adhesion mechanisms (8, 9). Low expression levels of ADAM23/MDC3 could cause defects in neuronal migration during brain development.

CREB1 (cAMP response element-binding protein 1) is involved in synaptic plasticity related to long-term memory (10) activating the cascade of proteins that participate in remodeling neuron processes (10, 11). The impairment of these remodeling events could be responsible for a reduction in dendritic outgrowth, necessary for the establishment of new synapses not only during brain development, but also during physiological reorganization of brain circuits that follows change in sensory stimulation and learning. KLF7 (Kruppel-like transcription factor 7) has a typical spatio-temporal pattern of expression in mouse. It is
highly expressed in spinal cord during the early embryonic period when motoneurons differentiate and in cortex in early postnatal period when a high rhythm of production of new synapses is observed (12). An impaired KLF7 expression could have a dramatic effect on the development of central nervous system, impairing correct differentiation of neuronal cells and production of new synapses.

Finally, the chromosome 2 deletion in our RTT-like patient encompasses MAP2 gene. This gene encodes for the microtubule-associated protein 2 (MAP2), a structural protein of developing brain dendrites. MAP2 reduces the critical concentration of tubulin required to polymerize microtubules and to maintain neuronal morphology (13). In RTT patients, alteration of neuronal maturation, dendritic anomalies (14–16), and decreased MAP2 immunoreactivity in white matter neurons are well documented (17). More recently, an expression profiling study performed on postmortem RTT brain showed decreased MAP2 expression. In particular, MAP2 expression is decreased more than 50% when compared with matched controls (18). The brain pathology in RTT reveals only subtle anomalies, in spite of the dramatic clinical deterioration observed in these patients (19). The neuropathological findings indicate that RTT may be a disorder due to an alteration of neuronal adaptive plasticity and to an arrest of neuronal development (20, 21). The dendritic alterations observed in postmortem tissue have been previously related to a reduction in the levels of MAP2 (22). MAP2, due to its role in maintaining neuronal morphology and in adaptive plasticity, could be a good candidate gene in the generation of features similar to RTT in our patient.

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4.4 2q24-q31 deletion: report of a case and review of the literature.


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2q24-q31 deletion: report of a case and review of the literature.

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ABSTRACT

We report a patient with a de novo interstitial deletion of the long arm of chromosome 2 involving bands 2q24.3-q31.1. The patient shows postnatal growth retardation, microcephaly, down-slanting palpebral fissures, long eyelashes and micrognathia. Halluces are long, broad and medially deviated, while the other toes are remarkably short with hypoplastic phalanges, and laterally deviated. She also showed developmental delay, seizures, lack of eye contact, stereotypic and repetitive hand movements and sleep disturbances with breath holding. Prenatal and three independent postnatal karyotypes was normal. Array-CGH analysis allow us to identify and characterize a “de novo” 2q interstitial deletion of about 10.4 Mb, involving segment between cytogenetic bands 2q24.3 and 2q31.1. The deletion was confirmed by quantitative PCR. About 52 children with 2q interstitial deletion have been reported. The deletion described here is overlapping with twelve of these cases. We have attempted to compare the clinical features of our patient with the twelve overlapping cases. The emerging phenotypes include low birth weight, postnatal growth retardation, mental retardation and developmental delay, microcephaly, and peculiar facial dysmorphisms. Peculiar long and broad halluces with an increased distance between the first and the second toe are present in all the described patients. The gene content analysis of the deleted region revealed the presence of some genes that may be indicated as good candidates in generating both neurological and dysmorphic phenotype in the patient. In particular, a cluster of SCNA genes is located within the deleted region and it is known that loss of function mutations in this gene cause a severe form of epilepsy.

KEY WORDS: Chromosome 2, mental retardation, digital anomalies, array CGH, 2q interstitial deletion.

INTRODUCTION

To date, more than 50 cases with a deletion of the long arm of chromosome 2 have been identified through standard cytogenetic analysis. The most frequent deletion involves the cytogenetic bands 2q31-q33 and corresponds to a specific phenotype [1] [2]. Deletion involving different segments of the long arm of chromosome 2 are characterized by a variability in breakpoints location and are clinically heterogeneous. Only few cases show overlapping deletions, although of quite different extensions. Moreover, most of the reported cases with 2q interstitial deletion were analysed with standard cytogenetic
techniques and shows a poorly definition of breakpoints. Given these data, genotype-phenotype correlations in cases with a 2q deletion not involving 2q31-q33 bands is more difficult.

We report here clinical description and molecular data of a new patient with a de novo 2q24-q31 deletion and compare his features with the phenotypes of other patients with overlapping deletions. The location of the chromosomal breakpoints and the size of the deleted region were identified by array-based Comparative Genomic Hybridisation (array-CGH) method.

**CASE REPORT**

The patient, a 4 years and 2 months old girl, is the third child of healthy unrelated parents. At birth, mother and father were 40 and 42 years old respectively. The two brothers, 19 and 14 years old, are healthy. Family history is unremarkable. No teratogen exposure during pregnancy was reported. The girl was born after 35 weeks of gestation. Apgar scores were 9 at 1 minute and 9 at 5 minutes, birth weight was 1950 g (10th-25th percentile), length was 45 cm (25th -50th percentile) and head circumference was 31 cm (25th percentile). At birth, clinical examination showed cleft palate, corrected at 11 months. At 6 months gastroesophageal reflux was diagnosed. Partial seizures, with secondary generalization, occurred at three months. EEG investigations showed slow background with high amplitude delta waves mixed with spikes and sharp waves on the temporo-occipital areas. She had a severe neuromotor developmental delay: she gained head control at 7 months, and she began to sit alone at 3 years of age. Presently, at the age of 4 years and 2 months, she is still unable to walk and speech is absent. Daily partial seizures occurred during the first years of life in spite of several antiepileptic drugs used in different therapeutic combinations. She was first visited by our medical unit at the age of 3.9 years. Her weight was 10 kg (-2.6 standard deviation scores), head circumference 43.5 cm (-2.7 standard deviation scores), and length 78 cm (-3 standard deviation scores).
At the physical examination she had hypotelorism, down-slanting palpebral fissures, long eye lashes, high nasal bridge with large nose, thick helices and ear lobule, mild micrognathia, cupid bow mouth, scialorrea, tapering fingers with clinodactyly of the 5th finger. Medially deviated, broad and long halluces are present. The other toes are deviated laterally, resulting in an increased distance between the first and the second toe. Other toes were remarkably short with hypoplastic phalanges. She also presented lack of eye contact, visual-motor coordination severely impaired, stereotypic and repetitive movements of the hands, bruxism and scialorrea. Sleep disturbances with breath holding were also noted.
The patient showed ipnic episodes of yell, tonic head and eye deviation towards the right side, followed by secondary generalization with tonic or tonic-clonic seizures. Moreover, flushing and hypertonia at the upper limbs with flexion of the right arm and extension of the contralateral arm were observed. Sometimes oral automatisms compare during the crisis. Sporadic myoclonic jerks were also present. In the clinical course, seizures became more polymorphic with both focal and generalized seizures. EEGs recorded slow background, sharp waves of high amplitude in the frontal region, spikes and spikes-waves in the fronto-central areas, bilaterally. The review of the EEG investigations, represented by interictal recordings, were consistent with the clinical semeiology. In fact, temporo-occipital paroxysmal activity was persistently recorded during the first year of life. Subsequently, epileptiform activity shifted towards the anterior areas (fronto-central regions) with evident secondary generalization. Seizure were resistant to several antiepileptic drugs in different combinations. Recently, the association of fenobarbital and levetiracetam was able to considerably reduce seizure frequency.

At the time of our second evaluation, at the age of 4 years and 2 months, growth parameters was unchanged. Development was still severely delayed and epilepsy remained uncontrolled by drug therapy.

Screening for neurometabolic disorders, organic acids, and aminoacids levels were normal or negative as well as serum and CSF lactate and pyruvate levels. MRI showed enlarged lateral ventricles and diffuse cortical atrophy associated with incomplete white matter myelination.

Fig. 2

Fig. 2 Hands and feet anomalies in the proband. A) View of the right hand showing the tapering fingers; B) View of the right foot showing the broad and long halluces and the increased distance between the first and the second toe. Clinodactyly of the 5th finger is also present.
Four independent karyotypes was performed, one prenatal e three postnatal executed in three different centers (major resolution been 550 bands). Sequence analysis of exons 2, 3 and 4 of the MECP2 gene showed no mutation.

**MATERIALS AND METHODS**

**Genomic DNA isolation, labelling and hybridization**

Genomic DNA of a normal female control was obtained from Promega. Genomic DNA of the patient was isolated from an EDTA peripheral blood sample by using a QIAamp DNA Blood Kit according to the manufacturer protocol (Qiagen, www.qiagen.com). We used the OD260/280 method on a photometer to determine the appropriate DNA concentration [3].

Ten micrograms of genomic DNA both from the patient (test sample) and from the control (reference sample) were sonicated. Test and reference DNA samples were subsequently purified using dedicated columns (DNA Clean and Concentrator, Zymo research, CA92867-4619, USA) and the appropriate DNA concentrations were determine by a DyNA Quant™ 200 Fluorometer (GE Healthcare, www.gehealthcare.com).

Array based CGH analysis was performed using commercially available oligonucleotide microarrays containing about 43,000 60-mer probes with an estimated average resolution of app. 35 kb (Human Genome CGH Microarray 44B Kit, Agilent Technologies). DNA labelling was executed essentially according to the Agilent protocol (Oligonucleotide Array-Based CGH for Genomic DNA Analysis 2.0v) using the Bioprime DNA labelling system (Invitrogen). Genomic DNA (2 μg) was mixed with 20 μl of 2.5X Random primer solution (Invitrogen) and MilliQ water to a total volume of 41 μl. The mix was denaturated at 95° C for 7 minutes and then incubated in ice/water for 5 minutes. Each sample was added with 5 μl of 10X dUTP nucleotide mix (1.2 mM dATP, dGTP, dCTP, 0.6 mM dTTP in 10 mM Tris pH 8 and 1 mM EDTA), 2.5 μl of Cy5-dUTP (test sample) or 2.5 μl of Cy3-dUTP (reference sample) and with 1.5 μl of Exo-Klenow (40 U/μl, Invitrogen). Labeled samples were subsequently purified using CyScribe GFX Purification kit (Amersham Biosciences) according to the manufacturer protocol. Test and reference DNA were pooled and mixed with 50 μg of Human Cot I DNA (Invitrogen), 50 μl of Blocking buffer (Agilent Technologies) and 250 μl of Hybridization buffer (Agilent Technologies). Before hybridization to the array the mix was denatured at 95° C for 7 minutes and then pre-associated at 37°C for 30 minutes. Probes was applied to the slide using an Agilent microarray hybridization station. Hybridization was carried out for 40 hrs at 65° in a rotating oven (20 rpm). The array was disassembled and washed according to the manufacturer protocol with wash buffers supplied with the Agilent 44B kit. The slides was dried and scanned using an Agilent G2565BA DNA microarray scanner.

**Image and data analysis**

Image analysis was performed using the CGH Analytics software v. 3.1 with default settings. 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 probe on the array to the oligonucleotide name, its position on the array and its position in the genome. The linear order of the oligonucleotides is reconstituted in the ratio plots consistent with an ideogram. The ratio plot is arbitrarily assigned such that gains and losses in DNA copy number at a particular locus are observed as a deviation of the ratio plot from a modal value of 1.0.

**Real-time quantitative PCR**

Real-time quantitative PCR was performed to confirm array-CGH data. We used an TaqMan Gene Expression Assays by design (Applied Biosystems, https://products.appliedbiosystems.com) which provides pre-designed primers-probe set for real-time PCR experiments. We designed the probe in the exon 2 of the Nostrin gene related to the 2q24.3 locus. Nostrin exon 2 forward primer: 5’-GCC AGA AAG TGG TGA TGC AAA-3’; Nostrin exon 2 reverse primer: 5’-CAA AGC TTG GAC TAA GTT C-3’; Nostrin exon 2 TaqMan probe: 5’-GCA GGC AGT GCA GAA CAG AT-3’. PCR was carried out using an ABI prism 7000 (Applied Biosystems) in a 96-well optical plate with a final reaction volume of 50 µl. A total of 100 ng (10 µl) was dispensed in each of the four sample wells for quadruplicate reactions. Thermal cycling conditions included a prerun of 2 min at 50°C and 10 min at 95°C. Cycle conditions were 40 cycles at 95°C for 15 sec and 60°C for 1 min according to the TaqMan Universal PCR Protocol (ABI). The TaqMan Universal PCR Master Mix and Microamp reaction tubes were supplied by Applied Biosystems. The starting copy number of the unknown samples was determined using the comparative Ct method as previously described [4].

**RESULTS**

The association of developmental delay, epilepsy and dysmorphic features prompted us to search for genomic rearrangements. Seventy-five kb resolution array-CGH experiments was performed. Despite the normal result of karyotyping, the analysis revealed a chromosome 2 interstitial deletion of 10.29-10.58 Mb (46,XX del (2)(q24.3-q31.1)) (Fig.2A). The proximal breakpoint is mapped in 2q24.3 (last oligonucleotide present located in 165.47 Mb, first deleted in 165.58 Mb position), while the distal breakpoint is located between 175.87 Mb and 176.05 Mb in 2q31.3 (last oligonucleotide deleted and first present, respectively). To confirm array data, Real-time Quantitative PCR experiments was performed in the patient and in her parents. The deletion was confirmed in the patient, while the parents showed a normal result (Fig.2B). Analysis of the gene content of the deleted region showed the presence of 50 known genes (Fig. 2C).
DISCUSSION

Even though the extension of the chromosome 2 interstitial deletion present in the patient (about 10 Mb) is significantly higher than the resolution limit of standard cytogenetic techniques, three independent chromosomal analyses failed to identify it. Prenatal diagnosis performed on the fetus with standard cytogenetic techniques (Q banding with an estimate resolution of about 320 bands) showed a normal female karyotype. After birth, given that the phenotype of the proband was strongly suggestive of a chromosomal imbalance the cyogenetic analysis was performed on three distinct peripheral blood samples. In all cases the karyotype was reported as female normal. The deletion was subsequently revealed by the use of array-based comparative genomic hybridization, a molecular cytogenetic technique with an extremely high resolution (about 75 kb). These data confirm the importance of a deeper investigation in patients in which the complex phenotype is strongly suggestive for the presence of a
chromosomal aberration and point at array-CGH as a powerful technique for the identification of rearrangements where conventional cytogenetic failed. The patient shows a de novo interstitial deletion of the long arm of chromosome 2, involving a segment of band 2q24.3 and a segment of band 2q31.1. About 52 children with 2q interstitial deletion have been reported [1] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14]. Among the reported cases, the following clinical manifestations are common: low birth weight, developmental delay and/or mental retardation, microcephaly and cleft palate. We have attempted to compare the deletion here reported with other previously described cases. Unfortunately, most of them are only cytogenetically characterized with poorly defined breakpoints. In spite of these limits, the deletion described here is overlapping with twelve cases previously reported (Table 1). Considering the twelve overlapping deletions, the emerging phenotype include low birth weight, postnatal growth retardation, mental retardation and developmental delay, microcephaly, down-slanting palpebral fissures, long eye lashes, micrognathia and low-set ears. Moreover, the presence of peculiar long and broad halluces with an increased distance between the first and the second toe is observed in 10 out of twelve patients (Table 1) and additional digital anomalies of hands and feet are present in all the reported cases. These anomalies include camptodactyly and clinodactyly of fifth finger, syndactyly of the fingertoes, duplicated halluces and hypoplastic third to fifth phalanges [10]. These features were first associated with the 2q24-q31 deletion by Moller et al. 1984. The presence in the reported case of medial deviation of the hallux and lateral deviation of the toes, brachysyndactyly, and clinodactyly of fifth finger confirm the association of digital anomalies with the 2q24-2q31 region. On the contrary, the vast majority of 2q deletion not overlapping this region usually does not show significant digital anomalies. The common limb phenotype in deletions involving the region 2q24-q31 suggests that genes in this area contribute to distal limb morphogenesis, according to Boles [10]. The gene content analysis of the deleted region shows the presence of 58 known genes. The cluster of genes HOXD3-HOXD13 is reported as good candidate for limb anomalies in patients with a 2q24-q31 deletion [15]. This cluster is excluded from the deleted region, however the patient shows tapering fingers with clinodactyly of the 5th finger, broad and long halluces with a wide gap between first and second toes, and shortness of the other toes that have also hypoplastic phalanges. The deleted region may contain regulatory elements for the HOXD genes that are located at a distance of about 0.8 Mb from the distal breakpoint [16]. The absence of such regulatory elements could explain the milder digital anomalies observed in the patient, with respect to other patients with a deletion including the HOXD cluster. Among the deleted genes, the DLX1 and DLX2 genes are included. The highly conserved Dlx1, 2, 5 and 6 homeobox transcription factors are involved in the regulation of basal ganglia and cortical local circuit neurons development [17]. Mice lacking Dlx1 show epilepsy due to the apoptotic death of cortical neurons and murine Dlx genes have a central role in controlling the development and function of forebrain GABAergic neurons [17] [18] [19]. The DLX genes are clustered on chromosome 2q (DLX1/DLX2) and on chromosome 7q (DLX5/DLX6). Different linkage
studies suggest that both these regions may be associated with autism [20], but sequence analysis of DLX genes in a cohort of 161 autistic probands failed in the identification of pathogenic mutations [21]. However, since these genes play a major role in control craniofacial patterning and differentiation and survival of forebrain inhibitory neurons [22] [23], DLX1/DLX2 haploinsufficiency might indeed be responsible for both neurological and dysmorphic phenotype in the patient.

A cluster of SCN genes including SCN3A, SCN2A2, SCN1A, SCN9A, and SCN7A is located within the deleted region. These genes codify for distinct alfa subunits of voltage-gated sodium channels and are involved in different types of epilepsy [24] [25] [26] [27]. Missense mutations in SCN1A are associate with GEFS+ (Generalized epilepsy with febrile seizures plus, OMIM#604233) a mild, dominantly inherited epilepsy. Non-sense and frameshift SCNA1 mutations cause SMEI (Severe Mioclonic Epilepsy of Infancy; OMIM#182389) that is often associated with mental deterioration [28]. The patient described here shows a peculiar electroclinical pattern with early-onset stereotyped seizures that became more polymorphic during the clinical course, with both focal and generalized seizures. The monosomy for SCN genes cluster may be responsible for the severe form of epilepsy observed in the patient, given that loss of function SCNA1 mutations in the heterozygous state are responsible for severe epilepsy phenotypes [29]. To establish whether an electroclinical pattern distinctive of patients with a 2q24-q31 deletion exists, clinical and EEG studies are necessary in other patients with overlapping deletions.

Considering literature data and the clinical and molecular features of the patient described here, we suggest to accurately evaluate the 2q24-q31 region in that cases presenting a syndromic condition with mental retardation, developmental delay, severe epilepsy and digital anomalies.

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| Table 1: Clinical findings in 234 patients with diabetes |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| CLINICAL FEATURES | Dk (200 mg/dL) | Rb (160 mg/dL) | Diab (200 mg/dL) | Diab (160 mg/dL) | Prox (50 mg/dL) | Distal (50 mg/dL) | Prox (50 mg/dL) | Distal (50 mg/dL) | Prox (50 mg/dL) | Distal (50 mg/dL) |
| Carcinoma       | 1               | 3               | 3               | 4               | 3               | 6               | 7               | 0               | 3               | 10              | 13              |
| Kasugai         | q40-q60         | q40-q60         | q40-q60         | q40-q60         | q40-q60         | q40-q60         | q40-q60         | q40-q60         | q40-q60         | q40-q60         | q40-q60         |
| JRA             | 7               | 7               | 7               | 7               | 7               | 7               | 7               | 7               | 7               | 7               | 7               |
| Age             | Young & old     | 20-70           | 40-60           | 60-80           | 80-100          | 100-120         | 120-140         | 140-160         | 160-180         | 180-200         | 200-220         |
| Blood glucose   | 100-200         | 110-160         | 120-180         | 180-240         | 240-300         | 300-360         | 360-420         | 420-480         | 480-540         | 540-600         | 600-660         |
| Weight          | +               | -               | +               | +               | +               | +               | +               | +               | +               | +               | +               |
| Height          | -               | -               | +               | +               | +               | +               | +               | +               | +               | +               | +               |
| Body mass index | +               | +               | +               | +               | +               | +               | +               | +               | +               | +               | +               |
| Blood pressure  | +               | +               | +               | +               | +               | +               | +               | +               | +               | +               | +               |
| Diastolic       | +               | +               | +               | +               | +               | +               | +               | +               | +               | +               | +               |
| Pulse           | 70-90           | 70-90           | 70-90           | 70-90           | 70-90           | 70-90           | 70-90           | 70-90           | 70-90           | 70-90           | 70-90           |
| Temperature     | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       |
| Blood pressure  | +               | +               | +               | +               | +               | +               | +               | +               | +               | +               | +               |
| Diastolic       | +               | +               | +               | +               | +               | +               | +               | +               | +               | +               | +               |
| Pulse           | 70-90           | 70-90           | 70-90           | 70-90           | 70-90           | 70-90           | 70-90           | 70-90           | 70-90           | 70-90           | 70-90           |
| Temperature     | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       | 36.5-37.5       |
* Heart murmur.
  a. Scaphocephaly, occipital meningomyelocele, internal hydrocephalus.
  b. Cataract, microphthalmia
  e. Occipital encephalocele, ventricular enlargement, lacunar skull defect.
  f. Craniosynostosis, proptosis.
  g. Sagittal synostosis, marked ridging of the sagittal suture, dysplastic nails of the halluces.
  h. Feet: broad and long halluces with a wide gap between first and second toes. Third, fourth and fifth digits remarkably short with hypoplastic phalanges.

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4.5 Retinoblastoma and associated malformations: characterization of three patients by array CGH


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Retinoblastoma and associated malformations: characterization of three patients by array CGH.

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ABSTRACT

We describe three patients with retinoblastoma, dysmorphic features and developmental delay. Case 1, a 1y 6m-old female, and case 2, a 2y 7m-old male, has a similar facial appearance with high and broad forehead, deeply grooved philtrum, and thick anteverted lobes and helix. Case 1 has dolicocephaly, sacral pit and toe crowding. Case 2 has severe intrauterine growth retardation and short fifth toe. Both case 1 and case 2 have partial agenesis of corpus callosum. Case 3 is a 7 year 6 months old female with mild to moderate growth retardation, severe microcephaly, thick lower lip and micrognatia. Array CGH demonstrates a de novo 13q deletion in cases 1 and 2. Case 3 has a 7q deletion inherited from the normal mother and probably not related to the disease. Our results confirm that there is a distinct facial phenotype related to 13q deletion contiguous gene syndrome characterized by high and broad forehead, deeply grooved philtrum and thick anteverted lobes. Patients with retinoblastoma and other malformations without a distinct facial phenotype may have a different contiguous gene syndrome or a casual association of mental retardation and retinoblastoma.

KEY WORDS: array CGH, deletion 13q syndrome, developmental delay, growth retardation, retinoblastoma.

INTRODUCTION

Retinoblastoma is the most common intraocular tumor in early childhood, with an incidence of 1/15,000-28,000 live births. Tumour development is caused by inactivation of both alleles of the RB1 gene located in 13q14.2. In the hereditary form (about 40% of RB cases), one of the mutation is transmitted as a dominant trait, by one of the parents. In sporadic case two mutational events have occurred in somatic cells. The vast majority of RB1 mutations reported in patients with retinoblastoma are point mutation, predominantly nonsense, frameshift or splicing mutations (1). RB1 gene deletion are found in a small part of cases (2). When the deletion involves a consistent part of surrounding genome it causes the so called 13q deletion syndrome, a contiguous gene syndrome characterized by retinoblastoma associated with developmental abnormalities. The first author that suggested a specific facial phenotype in 13q deletion was Motegi in 1983 (3). In 1999, Baud et al described a cohort of 22 patients and defined the dysmorphic
features of this syndrome (4). The most prominent dysmorphic abnormalities found in that cohort were anteverted ear lobes, high and broad forehead, and a prominent philtrum.

We present here three cases with retinoblastoma, dysmorphic features and developmental delay. The first two cases show an interstitial deletion on chromosome 13q and the third one has a proximal deletion on chromosome 7q inherited from the normal mother.

MATERIALS AND METHODS

Genomic DNA isolation, labelling and hybridization

Genomic DNA from normal male 46,XY and normal female 46,XX was obtained from Promega. Genomic DNA of the patients was isolated from an EDTA peripheral blood sample by using a QIAamp DNA Blood Kit according to the manufacturer protocol (Qiagen, www.qiagen.com). The Hoechest dye binding assay was used on a DyNA Quant™ 200 Fluorometer (GE Healthcare) to determine the appropriate DNA concentration.

Seven μg of genomic DNA both from the patient (test sample) and from sex-matched control (reference sample) were digested with AulI (10 units) and RsaI (10 units) restriction enzymes for 3h at 37°C. Test and reference DNA samples were subsequently purified using dedicated columns (DNA Clean and Concentrator, Zymo research, CA92867-4619, USA) and the appropriate DNA concentrations were determine by a DyNA Quant™ 200 Fluorometer.

Array based CGH analysis was performed using commercially available oligonucleotide microarrays containing about 43,000 60-mer probes with an estimated average resolution of about 75 kb (Human Genome CGH Microarray 44B Kit, Agilent Technologies). DNA labelling was executed essentially according to the Agilent protocol (Oligonucleotide Array-Based CGH for Genomic DNA Analysis 2.0v) using the Bioprime DNA labelling system (Invitrogen). Genomic DNA (2 μg) was mixed with 20 μl of 2.5X Random primer solution (Invitrogen) and MilliQ water to a total volume of 41 μl. The mix was denaturated at 95° C for 7 minutes and then incubated in ice/water for 5 minutes. Each sample was added with 5 μl of 10X dUTP nucleotide mix (1.2 mM dATP, dGTP, dCTP, 0.6 mM dTTTP in 10 mM Tris pH 8 and 1 mM EDTA), 2.5 μl of Cy5-dUTP (test sample) or 2.5 μl of Cy3-dUTP (reference sample) and with 1.5 μl of Exo-Klenow (40 U/μl, Invitrogen). Labeled samples were subsequently purified using CyScribe GFX Purification kit (Amersham Biosciences) according to the manufacturer protocol. Test and reference DNA were pooled and mixed with 50 μg of Human Cot I DNA (Invitrogen), 50 μl of Blocking buffer (Agilent Technologies) and 250 μl of Hybridization buffer (Agilent Technologies). Before hybridization to the array the mix was denatured at 95° C for 7 minutes and then pre-associated at 37°C for 30 minutes. Probes was applied to the slide using an Agilent microarray hybridization station. Hybridization was carried out for 40 hrs at 65° in a rotating oven (20 rpm). The array was disassembled and washed according to the manufacturer protocol with wash buffers supplied with the Agilent 44B kit. The slides was dried and scanned using an Agilent G2565BA DNA microarray scanner.

Image and data analysis

Image analysis was performed using the CGH Analytics software v.
3.1 with default settings. 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 linear order of the oligos is reconstituted in the ratio plots consistent with an ideogram. The ratio plot is arbitrarily assigned such that gains and losses in DNA copy number at a particular locus are observed as a deviation of the ratio plot from a modal value of 1.0.

Real-time quantitative PCR

Real-time quantitative PCR was performed to confirm array-CGH data. We used a TaqMan Gene Expression Assays by design (Applied Biosystems, https://products.appliedbiosystems.com) which provides pre-designed primers-probe set for real-time PCR experiments. In order to validate the presence of the 13q deletion in case 1 and case 2 we used the TaqMan probe and primers described in a previously report in the exon 17 of the RB1 gene (1). In order to validate the presence of 7q deletion in case 3 we designed the probe in the BC066990 sequence related to the 7q11 locus. Forward primer: 5’-GTG CTG TAG TGC AGA ATG TAA CAA A-3’; reverse primer: 5’-CAG AAA GCC AAG AAT AAC-3’; TaqMan probe: 5’-AGG GTG AAC AAA ACC AGT TGA GTT -3’. PCR was carried out using an ABI prism 7000 (Applied Biosystems) in a 96-well optical plate with a final reaction volume of 50 µl. A total of 100 ng (10 µl) was dispensed in each of the four sample wells for quadruplicate reactions. Thermal cycling conditions included a prerun of 2 min at 50°C and 10 min at 95°C. Cycle conditions were 40 cycles at 95°C for 15 sec and 60°C for 1 min according to the TaqMan Universal PCR Protocol (ABI). The TaqMan Universal PCR Master Mix and Microamp reaction tubes were supplied by Applied Biosystems. The starting copy number of the unknown samples was determined using the comparative Ct method as previously described (5).

RB1 mutation analysis

Genomic DNA was amplified by polymerase chain reaction (PCR). Primers and PCR conditions used for single exons and promoter analysis were as described previously (6) (7) (8). PCR products were mixed with an equal volume of formamide, denatured by heating at 95°C for 5 min followed by immediate chilling on ice. Single-strand conformational polymorphism (SSCP) was performed on a GenePhor apparatus (Pharmacia Amersham, Little Braunschweig, Germany) using a GeneGel Excel 12.5/24 Kit (Pharmacia Amersham).

RESULTS

Case 1

She is 1 year and 2 months old female (Fig.1A). She is the first and unique child of healthy unrelated parents. At birth, mother and father were 28 and 33 years old, respectively. Family history was unremarkable. No teratogen exposure during pregnancy was reported. The child was born on term with caesarean delivery. Birth weight was 3130 gr (50° percentile), length was 51 cm (50° percentile) and head circumference was 36 cm (>90° percentile). Bilateral retinoblastoma was diagnosed at 5 months. At that time, MRI revealed corpus callosum hypoplasia (Fig.2). ABR and ankle ultrasonography were normal. At our
first examination (6 months), psychomotor development was slightly delayed. Her weight was 7.850 g (75\textsuperscript{th} percentile), length 68 cm (90\textsuperscript{th} percentile) and head circumference 46 cm (97\textsuperscript{th} percentile) with dolicocephaly. The patient presented scalp anomalies including widely open fontanelles and an alopecic area on the right temporo-parietal region. High and broad forehead, deeply grooved philtrum, and thick anteverted lobes and thick helix were noted. In addition, she showed sacral dimple and clinodactyly of the 5\textsuperscript{th} toe on the left and toe crowding on the right (Fig.2A). At our second clinical examination (14 months) she showed: weight 9 Kg (10\textsuperscript{th}-25\textsuperscript{th} percentile), length 76-77 (50\textsuperscript{th} percentile), head circumference 49 cm (>97\textsuperscript{th} percentile). She had already surgical treatment of the right eye. Psicomotor delay persists. Ultrasound cardiac examination was normal (Tab. 1).

**Fig.1** Face and profile views of the patients. A) Case 1 at the age of 1y 2m. Frontal view showing high and broad forehead, deeply grooved philtrum. Side view showing dolicocephaly, thick anteverted lobes and helix. B) Case 2 at the age of 2y 7m. Frontal view showing hypotelorism, long palpebral fissures, epichantic folds, slight unilateral ptosis and thick and everted lower lip, in addition to case 1. Thick anteverted lobes and helix are noted on the side view. C) Case 3 at the age of 7y6m. Frontal view showing sparse eye-brows in the medial third, epichantic folds, broad nasal bridge, bulbous tip of the nose, long philtrum, thick and everted lower lip. Side view showing large ears and micrognathia.

**Case 2**
He is 2 years and 7 months old boy (Fig.1B). He is the third-born of healthy and non-consanguineous parents. Intrauterine growth retardation was noted at 36\textsuperscript{th} week of gestation. He was born on term. At birth, the weight was 2300 gr (<3\textsuperscript{rd} cnt), the lenght was 47 cm (10\textsuperscript{th}-25\textsuperscript{th} cnt) and the OFC was 32 cm (3\textsuperscript{rd} -10\textsuperscript{th} cnt). He presented a deficit of thermoregulation. At 11\textsuperscript{th} day he presented enterococcus sepsis. During first months of life parents noted bilateral heterochromia of iris. Right eye retinoblastoma was diagnosed unilateral at 10 months of age. At 1 year
of life a MRI was performed and hypoplasia of corpus callosum was noted. At our first examination (2 years of age) his height was 78 cm (<3rd cnt), weight was 8,250 Kg (<<3rd cnt); OFC was 44 cm (<<3rd cnt). He showed hypotonicity and particular facial features including high and broad forehead, deeply grooved philtrum, thick and everted lower lip, thick and everted auricular lobes, thick helix. Moreover, he had short 5th toe with hypoplasic toe nail (Fig.2B). Seven months later, a second clinical examination confirms growth delay: he was tall 80 cm, <5th cnt; weight was 9.0 Kg, <<3rd cnt, OFC was 45 cm, <<3rd cnt. The previously noted facial features were still present. There are no abnormalities of other organs and systems. He has reached self-governing deambulation at 2 years and 6 months. Presently, he is able to says only few words and he has not reached the sphincter control. An echocardiogram shows a minimum aortic reflux probably due to the infantile infection. An hands and toes X-ray examination does not indicate any abnormality (Tab. 1).

Fig. 2
Fig. 2 Toes anomalies in the patient 1 (A) and patient 2 (B). A) View of the right feet showing toe crowding. Note on the left feet clinodactyly of the 5th toe. B) Note short 5th toe with hypoplasic toe nail.

Case 3

She is 7 years and 11 months old female (Fig.3C). She is the first-born of healthy, nonconsanguineous parents. At the time of her birth, the mother was 35 and the father 47 years old. She has a 19 years old maternal half-sister, with suspect of Gilles De La Tourette syndrome. The mother had a spontaneous abortion at later time of her birth. During gestation, ultrasound study revealed microcephaly. She was born on term and her weight was 2,780 g (10th - 25th cnt), data on length and OFC are not avaible. Development has been slightly delayed: she has reached self-governing deambulation at 20 months of age and she said first words when she was 2 years old. At 2 ½ years of age, her mothers noted
right leucoria. It was diagnosed unilateral retinoblastoma, treated with eye enucleation. At our first examination (5 years and 7 months of age), her weight was 14 kg (< 5th cent), height was 109.5 cm (25-50th cent) and OFC was 41 cm (<< 3rd cent). Physical examination showed sparse eye-brows in the medial third, epicantthic folds, broad nasal bridge, bulbous tip of the nose, long philtrum, thick and everted lower lip, large ears, micrognathia and cutis marmorata. A second clinical examination at 7 years and 11 months of age confirmed short stature (115 cm, < 5th cent), the above described facial features and microcephaly (OFC 42 cm, <<3rd cent). She also showed a moderate mental retardation (Tab. 1).

### Table 1. Clinical features of the three cases.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>1y2m</td>
<td>2y7m</td>
<td>7y6m</td>
</tr>
<tr>
<td><strong>Dysmorphic features</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thick eyebrows</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thick, high and broad forehead</td>
<td>+</td>
<td>+</td>
<td>+/+/+</td>
</tr>
<tr>
<td>Deeply grooved philtrum</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Short nose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cardiac anomaly</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Brain anomaly</strong></td>
<td>Partial corpus callosum agenesis</td>
<td>Corpus callosum hypoplasia</td>
<td></td>
</tr>
<tr>
<td>** Skeletal abnormality**</td>
<td>Too crowded</td>
<td>Short W as</td>
<td></td>
</tr>
<tr>
<td><strong>Growth retardation</strong></td>
<td></td>
<td>+(&lt; 3 mg)</td>
<td>+/+</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td>Dolichocephaly</td>
<td>Aplagrotal</td>
<td>Intracranial</td>
</tr>
<tr>
<td><strong>Development delay</strong></td>
<td></td>
<td></td>
<td>+ (moderate mental retardation)</td>
</tr>
<tr>
<td><strong>Retinoblastoma characteristics</strong></td>
<td>5m</td>
<td>10m</td>
<td>2y5m</td>
</tr>
<tr>
<td><strong>Tumor</strong></td>
<td>Bilateral</td>
<td>Unilateral</td>
<td>Unilateral</td>
</tr>
</tbody>
</table>

**RB1 mutation analysis**

Point mutation analysis of the promoter and the coding sequence of the RB1 gene did not revealed any sequence variation.

**Array-CGH analysis**

Oligonucleotide array-CGH with an average spatial resolution of approximately 35 kb was performed on DNA of the three cases. The analysis of ratio profiles for case 1 and case 2 revealed an interstitial deletion in the long arm of chromosome 13 of different size. Based on the array findings, the deleted region of case 1 was found to extend approximately 19 Mb (46,XX del (13)(q13.3-21.2)). The proximal breakpoint is mapped in 13q13.3 (last oligonucleotide present located in 40.34 Mb, first deleted in 40.40 Mb position), while the distal breakpoint is located between 59.29 Mb and 59.36 Mb in 13q21.2 (last oligonucleotide deleted and first present, respectively) (Fig. 3A). The deleted region of case 2 is app. 36 Mb in size (46, XY del (13)(q14.11-31.1)). In this case, the proximal breakpoint is mapped in 13q14.11 (last oligonucleotide present located in 43 Mb, first deleted in 43.24 Mb position), while the distal breakpoint is located between 79.27 Mb and 79.80 Mb in 13q31.1 (last oligonucleotide deleted and first present, respectively) (data not shown). On the contrary, the array – CGH analysis of case 3 revealed a 200 Kb proximal deletion on chromosome 7q (46,XX del (7)(q11-21)) (Fig. 3B).
In order to confirm array findings, Real-time Quantitative PCR experiments was performed in the 3 patients and in their parents. In all three cases the deletion was confirmed (Fig.3D). Deletion of case 1 and 2 was de novo while deletion case 3 inherited from the unaffected mother (Fig.3D).

**Fig. 3** Molecular data of the patient. A) Chromosome 13 array CGH ratio profile using DNA from the case 1 and reference DNA from a normal female. On the left, the chromosome 13 ideogram. On the right, the log2 ratio of the chromosome 2 probes plotted as a function of chromosomal position. Oligo with a value of zero represent equal fluorescence intensity ratio between sample and reference. Each dot represents a single probe (oligo) spotted on the array. Copy number loss shift the ratio to the left (value of about -2X). B) Chromosome 7 array CGH ratio profile using DNA from the case 3 a reference DNA from a normal female. On the left, the chromosome 7 ideogram. C) and D) Real-time quantitative PCR validation experiment. C) RB1 ddCT ratios and standard deviations of three different controls and of the case 1 and 2 and their parents. The patients show ddCT ratio of about 0.5 the presence of a single copy of RB1 (deletion), while the parents as the controls show ddCT ratios of about 1.0, that indicates a double copy of the gene. D) BC066990 sequence ratios and standard deviations of three different controls and of the case 3 and her parents. The patient and mother show ddCT ratio of about 0.5 the presence of a single copy of BC066990 sequence (deletion), while the father show ddCT ratios of about 1.0, that indicates a double copy of the sequence.
DISCUSSION

Up to now, about 100 cases with 13q interstitial deletion have been reported (9). In 1995, Brown et al proposed a classification of 13q deletion syndrome based on the association between the extension of the deletion and the phenotypic features (10). According to this classification, cases with a deletion reaching band 13q32 show retinoblastoma associated with major malformations and digital abnormalities. Cases with smaller deletion not involving 13q32 are mildly affected. A more subtle characterization of this subgroup of patients was performed by Baud (4). Usually they have anteverted ear lobes, high and broad forehead, and a prominent philtrum (3) (11) (4).

Cases 1 and 2 have several facial features suggesting the 13q deletion described by Baud (Tab.1). In particular both patients show high and broad forehead, deeply groove philtrum, thick and anteverted lobes and thick helix. These characteristics are absent in patient 3. As expected on the basis of clinical examination, array CGH analysis confirm a 13q deletion syndrome in case 1 and 2 but not in case 3. Our findings confirm that a specific facial phenotype exist in 13q deletion syndrome and that this diagnosis maybe strongly suspected on clinical basis even before genetic test.

Gene content analysis of the minimal critical region deleted in patient 1 and 2 showed the presence of 39 known genes (Fig.4). Among them, NUFIP1 is of particular interest due to its putative role in central nervous system development and to its preferential expression in brain. The NUFIP1 gene encodes for a nucleocytoplasmatic RNA binding protein, the FMRP interacting protein 1. NUFIP1
interacts with FMRP, the protein disrupted in Fragile X Mental Retardation. The expression pattern of NUFIP1 and FMR1 are overlapping in brain (12). In particular, NUFIP1 is highly expressed in cortex, hippocampal neurons and cerebellar Purkinje cells. NUFIP1 could be involved in the regulation of local protein synthesis near active synapses in association with FMRP. Due to its role in synaptic plasticity NUFIP1 could be a good candidate gene for mental retardation in our patients.

A more complex situation is present in case 3. The presence of the small 7q deletion in the unaffected mother suggest that this deletion is not responsible for the clinical phenotype in this patient. Sharp et al (2005) already described a copy number polymorphism in the same region. In particular, they identify a duplication in 2 out of 47 normal individuals, using a segmental duplication targeted BAC array. These data strengthens the hypothesis that the inherited variant in the patient reported here may be a neutral genomic variation. However, analysis of 50 healthy subjects does not reveal similar deletion on chromosome 7. Therefore, the phenotype features of the patient remain unexplained and further analyses will be necessary to definitively rule out the involvement of this small deletion in the clinical phenotype of the patient.
REFERENCES


4.6 Array-CGH characterization of an additional patient with a 2q deletion

*Unpublished results*
Array – CGH characterization of an additional patient with a nonoverlapping 2q deletion

The patient is the male first child of unrelated parents. At the time of delivery the mother was 29-years-old and the father 28-years-old. The father refers a unilateral facial nerve palsy during the childhood and monolateral hypoacusia. The mother is healthy and the second child of the couple was a normal girl. During the pregnancy eclampsia occurred. The patient was born at 29 weeks gestational age. Birth weight was 1560 g (90th centile), length and OFC are not available. After birth the proband was hospitalised for about 10 weeks. He showed developmental delay. He reached head movements controls at 8 months of age and began to sit alone at 14 months and to walk independently at 22 months. Speech and language are still absent. Starting from the age of 7 years, he presented nocturnal apneas and sleep disturbances. He underwent surgery for unilateral cryptorchidism and then for repair unilateral inguinal hernia. Ophthalmologic examination, performed at the age of 3 years, revealed astigmatism, hypermetropia and intermittent exotropia. ABR index at the age of 4 years was normal. Brain MRI at 8 years revealed a mild enlargement of lateral ventricles. EEG investigation at the age of 7 years showed aspecific moderate abnormalities on centrals areas, and at the age of 9 years showed only disorganization of the background activity. Karyotype performed on a peripheral blood sample was reported as male normal.

Clinical examination at the age of 13 years and 8 months showed height 152 cm (10th to 25th centile), weight 54 kg (50th to 75th centile; the mother refers a marked increase after the introduction of the pharmacological therapy), OFC 57 cm (97th
centile). Father’s OFC is 61.5 cm (97th centile). The patient shows high forehead, thick and coarse hair, synophrys, an inner canthal distance of 3.8 cm (>2 SD), an outer canthal distance of 11 cm (+1/+2 SD), high palate, micrognathia. Teeth are broad with absence of right incisor. The right ear is dysmorphic with prominent anti-helix (Fig.1). Palm length is 9.5 cm (25th centile), middle finger length is 7.6 cm (50th centile), fingers are tapering with clubbing of the distal part. Foot length is 23.5 cm (3rd -25th centile) with bilateral sandal gap. The venous pattern is evident over the anterior thorax and the arms. Severe mental retardation and behavioural problems such as hyperactivity, screaming and periods of anxiety, aggressiveness and self mutilation are present.

At second examination, at the age of 14 years and 9 months, height was 160 cm (10th to 25th centile), weight 62 kg (75th centile), OFC 58 cm (>97th centile). Clinical and behavioural situation was unchanged.
Fig. 6 | **Dysmorphic features of the patients** at the age of 13 years and 8 months.

We performed array-CGH experiments by the use of an oligonucleotides array with an average spatial resolution of approximately 75 kb (Agilent Human Genome CGH Microarray 44B kit). The analysis revealed a chromosome 2 interstitial deletion of 12.75-13.47 Mb (46,XX del 2 (q31.2-q32.3)) (Fig.2). The proximal breakpoint is mapped in 2q31.2 (last oligonucleotide present located in 179.91 Mb, first deleted in 180.13 Mb position) and the distal breakpoint is located in 2q32.3between 192.88 Mb and 193.38 Mb (last oligonucleotide deleted and first present, respectively) (Fig. 6). To confirm array data, real-time quantitative PCR experiments was performed in the patient and in her parents. The deletion was confirmed in the patient, while the parents showed a normal result (data not shown).
Fig 7 | **Array-CGH characterisation of the chromosome 2 deletion.** Chromosome 2 array CGH ratio profile using DNA from the patient and reference DNA from a normal female. On the left, the chromosome 2 ideogram. On the right, the log2 ratio of the chromosome 2 probes plotted as a function of chromosomal position. Oligo with a value of zero represent equal fluorescence intensity ratio between sample and reference. Each dot represents a single probe (oligo) spotted on the array. Copy number loss shift the ratio to the left (value of about –2X).
5. DISCUSSION, CONCLUSION and FUTURE PERSPECTIVES
5. DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

The aim of this work was to investigate a selected group of patients with mental retardation and associated anomalies for the presence of submicroscopic chromosomal imbalances. Recently, several authors demonstrated that array-based Comparative Genomic Hybridisation is a powerful tool for the detection of cryptic chromosomal imbalances in patients with mental retardation and multiple congenital anomalies (MCA/MR). Consequently, we decided to employ this innovative methods to study our group of patients. We initially approached array-CGH using commercially available whole-genome coverage arrays with 1 Mb resolution. In a first phase of the study, we had several technical difficulties in optimising of the protocols and in choosing the appropriate devices for array-CGH. The importance of microarray scanner characteristics on experimental results is well documented. Unfortunately, the scanner that we have acquired in a first phase of the study it has been shown to be not suitable for BAC array-CGH applications, despite the statements of the manufacturer (Appendix 3).

Evaluating the performances of commercially available 1 Mb arrays, it appears evident that experimental and declared resolution are discordant. In fact, experimental resolution may be considerably lower than 1 Mb in particular for some regions of the genome, as the acrocentric chromosomes. As showed at page 29, using these arrays a five Mb deletion on chromosome 15 is pointed out by a single clone with a ratio value of about -0.5 in spite of the five expected. One of the criteria for the evaluation of results obtained with BAC arrays is that an aberration may be indicated by at least three consecutive clones with an aberrant ratio value. Consecutively, the deletion observed in this case should be missed employing only the 1Mb resolution array. On the basis of these technical observation and given that higher resolution platforms became rapidly available, we decided to employ oligonucleotide-arrays for our research purposes and we optimised array-CGH on tiling path oligonucleotide arrays with an average resolution of 75 kb, providing a full coverage of the human genome.
We have reported here the characterization of 5 patients that result from a pilot study involving a group of 15 patients with mental retardation and congenital anomalies. Among the five described patients, the male case with a 2q interstitial deletion was inserted within the Italian XLMR bank, a clinical and molecular database available at the web-address http://xlmr.unisi.it. The database was originally designed to collect both families with an X-linked pattern of inheritance of mental retardation and sporadic male cases with mental retardation with or without MCA. The collected patients have a normal standard karyotype and are negative for subtelomeric rearrangements (page 31). The first aim of the study was to perform mutation analysis for known mental retardation genes. Subsequently, the introduction of whole-genome array-CGH analysis allowed to establish that submicroscopic chromosomal imbalances are a major cause of hitherto idiopathic MCA/MR. Consequently, we decided to increase the number of sporadic cases collected in the database and to extensively apply whole-genome array-CGH at a resolution of 75 kb to analyze each case. The extensive use of array-CGH will contribute to unravel other autosomal mental retardation cases among those inserted in the database. The bank, enriched by these data, could become a resource also for autosomal MR. The careful clinical description that each patient must have to be inserted in the bank, combined to the accurate definition of chromosomal rearrangements obtained through array-CGH, will represent a unique resource for genotype-phenotype correlation studies and for the identification of emerging syndromes.

The use of the array-CGH approach allowed us to successfully characterized several patients with complex phenotypes and normal standard karyotype, confirming the power of array-CGH to identify and characterized chromosomal rearrangements in a single experiment, without previous information on their position and extension. In fact, the first case presented in this study, a patient with a Rett-like phenotype and a cytogenetically detectable 2q interstitial deletion, was studied using a classical approach with the employ of known polymorphic markers. Several experiments were necessary to map deletion breakpoints with a final resolution of 0.9 Mb. This approach is simple
and feasible, but it is labour and time consuming and its resolution power is limited by the number of available polymorphic markers. The introduction of array-CGH in our laboratory allowed us to overcome these limitations and to obtain the characterization of submicroscopic anomalies with higher resolution. In particular, we have described two additional patients with 2q interstitial deletions and a normal G-banded karyotype (pp. 47 and 70). The deletions have an extension of about 10.4 Mb and 12.25-13.47 Mb respectively, and the size of breakpoints extends from a minimum size of 0.1 Mb to a maximum of 0.5 Mb. Furthermore, it is important to note that in both cases the standard karyotypes were reported as normal, but the deletions are larger than the resolution limit of standard karyotype (5-10 Mb). In fact, detection limits of standard cytogenetics techniques are due not only to the size of the rearrangements but also to the chromosomal region involved, given that there are some chromosomal regions more difficult to investigate, and to the type of banding technique employed. Array-CGH allows to overcome also these limitations due to the intrinsic structural properties of chromosomes.

We have attempted a phenotypic comparison between the patient with the 2q24.3-2q31.3 deletion that we have described and other cases with overlapping deletions. Unfortunately, all the overlapping deletions already reported were characterized by standard cytogenetic techniques and breakpoints were poorly defined. Therefore, due to this more gross definition of the rearrangements the genotype-phenotype correlation results less precise. Consequently, it may be interesting, whenever possible, the re-evaluation by array-CGH of the reported overlapping rearrangements, to improve genotype-phenotype correlation studies of these cases.

Several studies have shown the potency of array CGH as a diagnostic tool. Very recently, Menten et al (2006) reported 1 Mb resolution array-CGH evaluation of 140 patients with mental retardation and multiple congenital anomalies. They identified chromosomal imbalances in about 20% of patients. The 13.5% of the imbalances were causative of the phenotype and, excluding that aberrations detectable also by subtelomeric screening, array-CGH analysis was able to identify the causative
aberration in the 9% of patients. Up to now, more than 400 patients with mental retardation and multiple congenital anomalies studied by array – CGH are reported in literature 44 62 67 72 73 74. Considering all the analysed patients, de novo causative chromosomal imbalances were detected in about the 9% of cases. In the 3% of cases parents were not available for the analysis. Finally about the 5% of patients showed an inherited chromosomal anomaly. At present, we have analyzed by tiling path array-CGH 15 patients with mental retardation and multiple congenital anomalies and we have identified four causative chromosomal deletions, with a detection rate comparable to that reported in literature.

In all reports the number of deletions is higher than duplications. These findings may have a technical cause due to the use of threshold algorithms that determine cut-offs for both deletions and duplications at equal distance from the mean of all intensity ratios. Since the intensity ratios for chromosomal deletions are more distant from the mean (ratio of 1/2) as compared with the intensity ratio observed for duplications (ratio of 3/2), inevitably there is a higher chance that some duplications may be missed 72. Furthermore, a clinical selection bias may exist, given that duplications generally result in a milder phenotype. In addition, the frequency of random duplication events in the human genome may be lower than the frequency of deletion events 75. Concordantly to these findings, in our preliminary study of 15 patients we have found four deletions and no duplications. To evaluate the relative contribute of genomic deletions and duplications in generating complex phenotypes, further studies in a larger cohort of patients also including milder phenotypes are necessary.

5.1 Array-CGH in a diagnostic setting

Several studies that includes large cohort of patients with MCA/MR have demonstrated that a high percentage of hitherto idiopathic MCA/MR is caused by submicroscopic chromosomal imbalances. More recently, the potential utility of array
CGH it is been demonstrated also in prenatal diagnosis 76. These results indicate that the screening of selected patients with normal standard karyotypes seems desirable and feasible. One of the main disadvantages to employ array-CGH in routine analysis are the high costs of the experiments. However, the development of an increasing number of commercially available platforms and of simplified laboratory protocols for labeling and hybridization procedures may have the effect to at least partially reduce both costs and time necessary to set up the technique. The increased knowledge on the importance of submicroscopic chromosomal rearrangements in MCA/MR and the technological improvements open the way for implementation of array-CGH in routine diagnostic analysis.

At present, it is debated which will be the optimal resolution for an array platform employed in a diagnostic setting to analyse MCA/MR patients. Higher resolution arrays may reveal higher numbers of chromosomal imbalances of less than 1 Mb in size, that are being missed with 1 Mb array. However, lower resolution arrays may guarantee a decreased false positive rate, especially if the identification of imbalances is based on alteration of the ratio values for three or more flanking clones. In addition, particularly in light of emerging information about large copy number variation in normal human populations, high resolution arrays are likely to generate some difficulties in interpretation 77 76 78. Considering the large percentage of inherited chromosomal imbalances, establishing benign copy number variations in the human genome will be of major importance in order to understand which imbalances are causative.

The incidence of small imbalances in different groups of patients is still unknown. More studies by high resolution array-CGH are necessary to establish whether higher resolution really increases the diagnostic yield in this patients and to evaluate which are the better resolution and configuration of an array for clinical use. The difficult process of introducing array-CGH into diagnostic settings requires that molecular approaches are supported by a well defined clinical characterization of
patients. Only this continuous exchange of information between these two approaches will guarantee the appropriate molecular analysis for each single patient.
6. REFERENCES
6. REFERENCES


7. APPENDIX 1
Chromosomal alterations detected by comparative genomic hybridization in nonfunctioning endocrine pancreatic tumors

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Abstract

We have used comparative genomic hybridization to investigate changes in DNA copy number in 20 nonfunctioning endocrine pancreatic tumors. The total number of regional DNA imbalances per tumor showed variation from case to case and high genetic heterogeneity was observed. From 1 to 22 chromosomal anomalies were detected in 13 of the 20 cases evaluated. Overall gains predominated over losses with a ratio of about 3:9:1 (58 gains/15 losses). The mean total number of regions displaying imbalances increased from 1.25 per tumor for benign tumors to 5.25 for malignant tumors, although statistical significance was not reached (\(P = 0.074\)). © 2005 Elsevier Inc. All rights reserved.

1. Introduction

Pancreatic endocrine tumors (PET) are rare neoplasms arising from pancreatic islet cells. Their annual incidence is approximately five cases per million [1] and accounts for less than 1% of all pancreatic neoplasms [2]. Four genetic syndromes have been reported to be associated with PET: multiple endocrine neoplasia type I (MEN-I), von Hippel-Lindau, von Recklinghausen’s disease (NF-I), and tuberous sclerosis [2]. The presence of metastases, especially in the liver, is the most important determinant of survival. The 5-year survival rate is 20–50% in patients with liver metastases and increases to 65–100% in those without liver metastases [1]. Despite recent advances made at the molecular genetic level, the molecular pathogenesis of PET is not well understood (for review see Gumbs et al. [3]). In particular, little is known about the genetic changes associated with metastatic dissemination in PET. A better understanding of the molecular mechanisms of metastatic progression could help to improve therapy in these patients [1].

The majority of PET are functionally active from a clinical standpoint and give rise to specific syndromes because of excess secretion of hormone, the most common of which are insulinomas [4]. Nonfunctioning PET (NF-PET) do not cause a distinct clinical syndrome because there is no hormonal hyperscetion [1]. NF-PET usually have a less favorable clinical outcome than clinically functioning PET [5] and are malignant in up to 92% of cases, compared with 50–60% of gastrinomas or 10% of insulinomas [6].

The objective of the present study was to characterize the genetic alterations of 20 NF-PET that were subgrouped into malignant and benign classes and to correlate these results with clinicopathologic data.

2. Materials and methods

2.1. Tumor specimens

Twenty NF-PET diagnosed at the Department of Pathology, University of Verona, were included in this study. Clinicopathologic data are summarized in Table 1. Eight cases were benign because they were confined to pancreatic structures. Twelve tumors were malignant, 11 had nodal and/or hepatic metastases, and 1 malignant case had infiltration of surrounding organs without metastases. Only one case was associated with MEN1 syndrome (Table 1). The majority of
patients were females, with a male/female ratio of approximately 1:1.5. The mean patient age was 54.4 years (range 32–78). Eleven samples were paraffin-embedded while nine were from frozen tissue (Table 1).

### Table 1
Clinicopathological data in 29 nonfunctional pancreatic endocrine tumors

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Ben/Mal</th>
<th>Size (cm)</th>
<th>Local invasion</th>
<th>Metastases</th>
<th>Follow-up (mo)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFP</td>
<td>F</td>
<td>47</td>
<td>Mal</td>
<td>Multiple nodules</td>
<td>Spleenic and mesenteric veins</td>
<td>Pancreatic LN, liver</td>
<td>24</td>
<td>Died of disease</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;2&lt;/sup&gt;</td>
<td>M</td>
<td>48</td>
<td>Mal</td>
<td>6</td>
<td>Peripancreatic fat, spleen</td>
<td>LN and liver multiple</td>
<td>54</td>
<td>Died of disease</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;3&lt;/sup&gt;</td>
<td>M</td>
<td>58</td>
<td>Mal</td>
<td>7</td>
<td>Peripancreatic fat, spleen</td>
<td>Pancreatic LN, liver</td>
<td>38</td>
<td>Died of disease</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;4&lt;/sup&gt;</td>
<td>F</td>
<td>55</td>
<td>Mal</td>
<td>6</td>
<td>Spleenic vein</td>
<td>Liver</td>
<td>95</td>
<td>Alive with disease</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;5&lt;/sup&gt;</td>
<td>F</td>
<td>56</td>
<td>Mal</td>
<td>10</td>
<td>Spleenic vein</td>
<td>Liver</td>
<td>37</td>
<td>Died of disease</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;6&lt;/sup&gt;</td>
<td>F</td>
<td>62</td>
<td>Mal</td>
<td>2</td>
<td>No</td>
<td>Pancreatic LN, liver</td>
<td>44</td>
<td>Alive with disease</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;7&lt;/sup&gt;</td>
<td>F</td>
<td>43</td>
<td>Mal</td>
<td>3</td>
<td>Peripancreatic fat</td>
<td>Pancreatic LN</td>
<td>83</td>
<td>Alive and well</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;8&lt;/sup&gt;</td>
<td>M</td>
<td>78</td>
<td>Mal</td>
<td>3</td>
<td>Peripancreatic fat</td>
<td>Pancreatic LN</td>
<td>6</td>
<td>Died of disease</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;9&lt;/sup&gt;</td>
<td>M</td>
<td>38</td>
<td>Mal</td>
<td>6</td>
<td>Mesenteric vein</td>
<td>Peripancreatic LN</td>
<td>90</td>
<td>Died of disease</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;10&lt;/sup&gt;</td>
<td>M</td>
<td>57</td>
<td>Mal</td>
<td>7</td>
<td>Peripancreatic fat, splenic vein</td>
<td>Peripancreatic LN</td>
<td>70</td>
<td>Alive with disease</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;11&lt;/sup&gt;</td>
<td>F</td>
<td>65</td>
<td>Mal</td>
<td>3</td>
<td>Peripancreatic fat</td>
<td>Peripancreatic LN</td>
<td>48</td>
<td>Died of disease</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;12&lt;/sup&gt;</td>
<td>F</td>
<td>54</td>
<td>Mal</td>
<td>2</td>
<td>Duodenum</td>
<td>No</td>
<td>114</td>
<td>Alive and well</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;13&lt;/sup&gt;</td>
<td>M</td>
<td>67</td>
<td>Ben</td>
<td>1.5 (cystic)</td>
<td>No</td>
<td>No</td>
<td>62</td>
<td>Alive and well</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;14&lt;/sup&gt;</td>
<td>F</td>
<td>49</td>
<td>Ben</td>
<td>11</td>
<td>No</td>
<td>No</td>
<td>66</td>
<td>Alive and well</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;15&lt;/sup&gt;</td>
<td>F</td>
<td>61</td>
<td>Ben</td>
<td>3.5</td>
<td>No</td>
<td>No</td>
<td>125</td>
<td>Alive and well</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;16&lt;/sup&gt;</td>
<td>F</td>
<td>47</td>
<td>Ben</td>
<td>2</td>
<td>No</td>
<td>No</td>
<td>34</td>
<td>Alive and well</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;17&lt;/sup&gt;</td>
<td>M</td>
<td>44</td>
<td>Ben</td>
<td>2.5</td>
<td>No</td>
<td>No</td>
<td>130</td>
<td>Alive and well</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;18&lt;/sup&gt;</td>
<td>M</td>
<td>57</td>
<td>Ben</td>
<td>1.5</td>
<td>No</td>
<td>No</td>
<td>95</td>
<td>Alive and well</td>
</tr>
<tr>
<td>NFP&lt;sup&gt;19&lt;/sup&gt;</td>
<td>F</td>
<td>70</td>
<td>Ben</td>
<td>3</td>
<td>No</td>
<td>No</td>
<td>62</td>
<td>Alive and well</td>
</tr>
</tbody>
</table>

Abbreviations: LN, lymph nodes; Ben, benign; Mal, malignant.
* Frozen tumor samples.
* Paraffin-embedded cases.

### 2.2. DNA extraction

Tumor DNA from paraffin-embedded or frozen tumor material and reference DNA from blood lymphocytes were extracted according to standard protocols [7].

### 2.3. Comparative genomic hybridization (CGH)

Target metaphase spreads were prepared according to standard protocols using phytohemagglutinin-induced peripheral blood lymphocytes from healthy volunteers. Metaphase chromosome preparations suitable for CGH were chosen by examining slides under a phase contrast microscope. Slides with a high mitotic index, little or no cytoplast, and minimal overlapping were selected [8].

Tumor DNA (1 μg) was labeled with biotin-DUTP in a nick-translation reaction. Normal reference DNA was labeled with digoxigenin-DUTP using the same procedure. The fragment length range was adjusted to 500-1500 base pairs by optimizing the labeling reaction conditions. Approximately 400 ng each of labeled tumor and labeled reference DNA were cohybridized with 40 μg of human Cot 1 DNA and 5 μg salmon sperm DNA, and the resulting pellet was dissolved in 10 μL buffer [50% formamide, 2× standard saline citrate (SSC), 20% dextran sulphate, pH 7], as described by Tarasucio et al. [9]. This probe was denatured at 75°C for 10 minutes and preannealed at 37°C for 20 minutes. The target slides contained normal metaphase cells and were denatured at 70°C for 2 minutes in 70% formamide and 2× SSC. The hybridization was carried out at 37°C for 3 days. Posthybridization washes were done in 50% formamide/2× SSC at 45°C (3×5 minutes) and 0.1×SSC at 60°C (3×5 minutes). After the posthybridization washes and detection, the slides were dehydrated in a series of 70%, 80%, and 95% ethanol. Air-dried slides were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in antifade mounting solution (Vector Laboratories, Burlingame, CA).

### 2.4. Allelic loss analysis

Allelic loss analysis and evaluation were performed essentially as described previously [10,11]. The primers used were from the ABI Prism Linkage Mapping Set version 1 or 2 (Applied Biosystems, Foster City, CA) as described [10,11]. Polymerase chain reaction products were pooled and electrophoresed on an ABI 377 DNA sequencer. Only microsatellites showing two distinct alleles in normal DNA were considered informative. For these, the intensity of the fluorescent bands of tumor was compared with that of matched normal DNA. Loss of heterozygosity (LOH) was scored when there was at least a 90% difference in the ratio of the intensities of the two alleles in tumor and matched normal DNA. Allelic imbalance was scored for those microsatellite markers showing at least a 50% difference in the ratio of the intensities of the two alleles in tumor and matched normal DNA.

### 2.5. Digital image analysis

Gray level images were acquired for each fluorescent dye with a charged coupled device camera on a Zeiss Axioskop
epiluorescent microscope (Carl Zeiss, Jena, Germany) using Vysis software and hardware (Vysis, Downers Grove, IL). Chromosomes were identified using reverse DAPI banding.

The green/red ratio of each entire metaphase was normalized to 1. Five to 12 metaphase spreads were analyzed from each tumor sample. Data were combined to generate an average ratio profile. Chromosomal regions were interpreted as gained when the red/green profile ratio exceeded 1.25, highly amplified when the ratio exceeded 1.5, and lost when the ratio was less than 0.75. As suggested by validation procedures of the technique, we excluded the Y chromosome and the centromeric, heterochromatic, and telomeric regions [8,9].

2.6. Statistical analysis

Student's t-test was used to evaluate the statistical significance in the difference among the mean total number of anomalies detected in malignant and benign tumors ($P = 0.074$).

3. Results

Chromosomal anomalies were detected in 13 of the 20 PET (65%). The chromosomal localization of DNA gains and losses is given in Table 2, and an example of the green/red CGH ratio peak profile belonging to case NF9 is shown in Fig. 1. A summary of the genomic imbalances detected in the 20 samples is given in the ideogram in Fig. 2. Overall, 73 anomalies were detected with a mean of 3.65 per case. Gains predominated over losses with a ratio of approximately 3.9:1 (58 gains/15 losses). The total number of regional DNA imbalances per tumor showed variation from case to case. Case NF10 displayed the largest number of changes, involving 22 chromosomal regions; case NF24 had the lowest number of changes, involving only 1 region. Seven cases did not show any anomaly (Table 2). The genomic imbalances included the following: (1) two 11p13 gains and one high-amplification observed in 3 out of the 20 cases (15%), all of them malignant; (2) a 9q21 gain observed in three cases (15%), two of which were malignant and one benign; (3) 6q25 and 6q27 gain both observed in two cases (10%), one malignant case and one benign; and (4) one 8q43.3 gain and one high amplification in two malignant cases (10%).

Overall high-level amplifications were detected in six cases, four malignant and two benign. NF9 and NF17 showed high-level amplifications in 13q32–q34, 18q11.2 and 9q21, 11p12–p14, respectively. Four cases showed only one high-level amplification (Table 2).

The number of underrepresented regions was much lower than that of overrepresented regions. The mean total number of regions displaying imbalances per tumor was different between the malignant and benign groups. In fact, we observed 63 anomalies in 12 patients with a malignant tumor (mean, 5.25 per case) and 10 anomalies in 8 patients with benign tumors (mean, 1.25 per case), although the difference was not statistically significant (t-test, $P = 0.074$).

![Table 2]

Summary of CGH data in 20 nonfunctional pancreatic endocrine tumors

<table>
<thead>
<tr>
<th>Case</th>
<th>Ben/Mal</th>
<th>Gains</th>
<th>High-level amplifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF9</td>
<td>Mal</td>
<td>None</td>
<td>5p, 5cen-q23, 3q31–q33, 7p, 7cen-q36, 9q21-q34, 13cen-q34, 15cen-q21, 15q25–q26, 18p, 18cen-q23, 20p, 20cen-q11.2, 20q12–q13.1, 20q13.3</td>
</tr>
<tr>
<td>NF27</td>
<td>Mal</td>
<td>7q26, 22q13.3</td>
<td>5q35.3, 8q24.3, 11p13, 15q14–q21, Xq27–28</td>
</tr>
<tr>
<td>NF29</td>
<td>Mal</td>
<td>1q43, 3q28, 6p24–q25, 11q24–q25, 18p11.3, 22q13.3</td>
<td>7q11.2–q21, 9q13–q21</td>
</tr>
<tr>
<td>NF24</td>
<td>Mal</td>
<td>None</td>
<td>8q24.3</td>
</tr>
<tr>
<td>NF18</td>
<td>Mal</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>NF20</td>
<td>Mal</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>NF6</td>
<td>Mal</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>NF10</td>
<td>Mal</td>
<td>3p23–q26, 3p14–p21, 11q23, 11q24, 16q21</td>
<td>1cen-q23, 1q25–q31, 1q32, 1q41–42, 2q33, 3q25, 6p22, 6q15, 6q24–25, 8q13, 8q21–22, 8q22–q23, 9q23, 10q23, 11p11.2–p14, 18p, 19cen-q13.1</td>
</tr>
<tr>
<td>NF17</td>
<td>Mal</td>
<td>18q23</td>
<td>9q13–q21, 11p12–p14</td>
</tr>
<tr>
<td>NF25</td>
<td>Mal</td>
<td>None</td>
<td>20p11.2–p12, 20q11.2</td>
</tr>
<tr>
<td>NF26</td>
<td>Mal</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>NF28</td>
<td>Mal</td>
<td>None</td>
<td>3q28–q29, 6q27, 19p11.2, 19q13.1, Xq25–q26</td>
</tr>
<tr>
<td>NF23</td>
<td>Ben</td>
<td>None</td>
<td>9q13–q21, 13q4, 15q11–q13</td>
</tr>
<tr>
<td>NF2</td>
<td>Ben</td>
<td>None</td>
<td>6q25, 6q27</td>
</tr>
<tr>
<td>NF4</td>
<td>Ben</td>
<td>None</td>
<td>3q28–q29, 4q34–35</td>
</tr>
<tr>
<td>NF30</td>
<td>Ben</td>
<td>9q13</td>
<td>None</td>
</tr>
<tr>
<td>NF5</td>
<td>Ben</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>NF19</td>
<td>Ben</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>NF1</td>
<td>Ben</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>NF3</td>
<td>Ben</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

**Abbreviations:** Ben, benign; Mal, malignant.
Microsatellite analysis on selected cases confirmed the results of CGH. The results are shown in Fig. 3, where both loss of heterozygosity and allelic imbalances that may be indicative of genomic amplification can be observed [11].

4. Discussion

Previous molecular studies have shown a significant molecular heterogeneity among PET. The NF-PET allelotype is quite different from that of exocrine tumors, as well as from that of functioning PET [5,12]. Nonfunctioning pancreatic tumors frequently have mutations in the MEN1 gene and may be further subdivided into two clinically relevant subgroups on the basis of the amount of chromosomal alterations. One group shows frequent and large losses, while the other shows a small number of random losses [5]. Chromosomes 6q, 11, 20q, and 21 show frequent loss of heterozygosity [12].

Moreover, a high frequency of large allelic losses is correlated with aneuploidy, while a small number of random losses is associated with diploidy [12]. Allelic loss in chromosone 22q12 was found at a high frequency in both functioning PET and NF PET; the loss size was associated with tumor aggressiveness [6].

Few CGH studies of NF-PET have been reported (Table 3) [1,13–15]. Overall, high genetic heterogeneity and involvement of all chromosomes has been found, while the loss/gain ratio varied from 1:1.2 to 1:3.5 in the different studies. The average number of anomalies in malignant tumors was higher than in benign tumors. It has been proposed that specific chromosomal regions could be involved in NF-PET tumorigenesis and/or malignancy (Table 4).

In our analysis, we observed a genetic heterogeneity and a predominance of gains; moreover, the mean total number of regions per tumor displaying imbalances differed between the malignant and benign groups. Among the few losses we observed, the chromosome 22q13.3 region was lost in two malignant cases, both with metastases in the regional lymph nodes and liver. It has been reported that allelic loss of 22q is positively correlated with distant metastases in PET [6]. These findings are suggestive of novel tumor suppressor gene loci at chromosome 22q that might contribute to the pathogenesis of PET, especially to the development of distant metastases [6]. Inactivation of a tumor suppressor gene in 22q13.3 is also suggested to play a role in the malignant progression of other tumor types, including astrocytoma, colorectal, ovarian, and breast cancer [16].

We did not detect losses on 6q, which have been reported by Speel et al. [14] as a frequent event, and we observed a
loss in 6p24–p25, a region containing tumor suppressor genes [17], in only one malignant case. These findings emphasize the cytogenetic heterogeneity of PET.

Chromosomal regions 6q25 and 6q27 showed gains in two cases, one malignant and the second benign. In the latter, 6q27 was highly amplified. The widely expressed GPR31 (G protein–coupled receptor 31, Mendelian Inheritance in Man (MIM) no. 602043) gene is located in 6q27 [18]. The estrogen receptor 1 gene (ESR1 gene) is localized in 6q25, has extensive homology with the erb-A protein of the oncogenic avian erythroblastosis virus [19], and is known to be expressed in the pancreas. The steroid hormones

Fig. 2. Gains and deletions detected in 20 nonfunctioning endocrine pancreatic tumors. Each of the bars on the right represents a copy number gain in a tumor, and each bar on the left represents a deletion.

Fig. 3. Representative electropherogram tracings of microsatellite analysis. Marker names and case numbers are indicated along with examples showing allelic loss (A and C) and allelic imbalances (B and D). N, normal; T, tumor.
and their receptors are involved in the regulation of endocrine gene expression, and they affect cellular proliferation and differentiation in target tissues [20]. The *MAS5* protooncogene (MIM no. 165180) has also been mapped to the 6q25 region. Young et al. [21] suggested that the *MAS1*-encoded protein may be a receptor that, when activated, modulates a critical component in a growth-regulating pathway that triggers its oncogenic effects [22]. It can be suggested that gain of the 6q25 and 6q27 regions is correlated with cellular proliferation through overexpression of one or more of these genes.

A common region of gain that was detected in three malignant cases was 11p13, where the *WTI* gene (MIM no. 603702) is located. In one case, this region was highly amplified. The *WTI* gene encodes a zinc finger DNA-binding protein that acts as a transcriptional activator or repressor, depending on the cellular or chromosomal context. *WTI* is required for normal formation of the genital urinary system and mesothelial tissues [22], and mutations are reported in diverse tumor types [23]. Although originally identified as a tumor suppressor gene, *WTI*, both wild type and mutant, is overexpressed in a variety of hematologic malignancies and solid tumors [24].

We detected one 9q21 gain and two 9q21 high amplifications in one benign and two malignant cases. It can be speculated that this region is not involved in PET malignancy.

The 19q13.1 region was gained in two cases, both of them malignant. This chromosomal region contains the *AKT2* oncogene (MIM no. 164731), which is overexpressed

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex/age</th>
<th>Diagnosis</th>
<th>Size (cm)</th>
<th>Gains</th>
<th>Losses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>M/53</td>
<td>Metastatic</td>
<td>3.7</td>
<td>4, 5, 7, 9, 12, 13q, 17q, 18p, 18q, 20, X</td>
<td>1, 2, 3, 6, 8, 10, 11, 15q, Y</td>
</tr>
<tr>
<td>2*</td>
<td>M/67</td>
<td>Metastatic</td>
<td>5</td>
<td>4p, 7q, 17q, 18, 20q</td>
<td>2q, 3, 6q, 13q14-qter</td>
</tr>
<tr>
<td>3*</td>
<td>M/49</td>
<td>Metastatic</td>
<td>5</td>
<td>4p, 4q12q25, 5q, 7q, 9p23-pter, 9q, 12, 13q, 14q, 17q, 18p, 18q11q12, 20q</td>
<td>2q, 6, 8, 10</td>
</tr>
<tr>
<td>4*</td>
<td>M/70</td>
<td>Metastatic</td>
<td>5</td>
<td>5q12q23, 8q, 12q12q21</td>
<td>1, 2, 4q22-qter, 6, 10, 11, 15q, 16</td>
</tr>
<tr>
<td>5*</td>
<td>F/64</td>
<td>Metastatic</td>
<td>5</td>
<td>17q, 20q</td>
<td>14q41, 5q, 6, 10, 11, 14q24-qter, 21q, X</td>
</tr>
<tr>
<td>6*</td>
<td>M/60</td>
<td>Metastatic</td>
<td>5.2</td>
<td>4, 5, 14q12q21, 17, 18</td>
<td>2q, Y</td>
</tr>
<tr>
<td>7*</td>
<td>F/64</td>
<td>Metastatic</td>
<td>10</td>
<td>7, 12q24, 14q, 17p, 17p</td>
<td>3, 6q, 8q, 10, 11, X</td>
</tr>
<tr>
<td>8*</td>
<td>M/34</td>
<td>Metastatic</td>
<td>10</td>
<td>4p, 5p, 9p31qter, 12q23pter</td>
<td>None</td>
</tr>
<tr>
<td>9*</td>
<td>M/61</td>
<td>Metastatic</td>
<td>1</td>
<td>64</td>
<td>None</td>
</tr>
<tr>
<td>10*</td>
<td>F/77</td>
<td>Metastatic</td>
<td>0.9</td>
<td>11</td>
<td>None</td>
</tr>
<tr>
<td>11*</td>
<td>F/51</td>
<td>Nonmetastatic</td>
<td>1.2</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>12*</td>
<td>F/72</td>
<td>Nonmetastatic</td>
<td>2</td>
<td>8q21pter, 12q24, 20</td>
<td>None</td>
</tr>
<tr>
<td>13*</td>
<td>F/50</td>
<td>Nonmetastatic</td>
<td>2.4</td>
<td>17q</td>
<td>8q</td>
</tr>
<tr>
<td>14*</td>
<td>F/70</td>
<td>Nonmetastatic</td>
<td>1</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>15*</td>
<td>F/70</td>
<td>Benign</td>
<td>1</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>16*</td>
<td>F/51</td>
<td>Benign</td>
<td>1.2</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>17*</td>
<td>M/77</td>
<td>Benign</td>
<td>0.9</td>
<td>None</td>
<td>11p</td>
</tr>
<tr>
<td>18*</td>
<td>M/55</td>
<td>Benign</td>
<td>1.8</td>
<td>None</td>
<td>11p, Y</td>
</tr>
<tr>
<td>19*</td>
<td>M/61</td>
<td>Malignant</td>
<td>1</td>
<td>6q, 7q22-ter</td>
<td>None</td>
</tr>
<tr>
<td>20*</td>
<td>F/17</td>
<td>Malignant</td>
<td>2</td>
<td>None</td>
<td>3q, 6q, 9q, 13q, Xp</td>
</tr>
<tr>
<td>21*</td>
<td>M/62</td>
<td>Benign</td>
<td>0.6</td>
<td>4q, 5p5, 7q11.2, 7q32-36, 12q24, 13q14q23-ter, 17p, Y</td>
<td>6q</td>
</tr>
<tr>
<td>22*</td>
<td>M/70</td>
<td>Benign</td>
<td>1.1</td>
<td>4q, 7q, 10q21.3-ter, 20q, 21q, Y</td>
<td>6q12-23, 13q21-23</td>
</tr>
<tr>
<td>23*</td>
<td>F/72</td>
<td>Malignant</td>
<td>2</td>
<td>4p13-q24, 8p21-ter, 12q13.3-ter, 14q21-ter, 18q, 20q</td>
<td>1p21-31.1, 6q22-22.1</td>
</tr>
<tr>
<td>24*</td>
<td>M/71</td>
<td>Malignant</td>
<td>2</td>
<td>4p15-ter, 9q22-ter, 14q24.3-ter, 17q, 20q</td>
<td>1p21-31.1, 2q14-35, 3q, 6q, 11p12-14.2, 11q14-ter</td>
</tr>
<tr>
<td>1*</td>
<td>M/50</td>
<td>No lymph node and no liver metastasis</td>
<td>Not reported</td>
<td>4q15pter, 17q, 18q12q13pter, 22q</td>
<td>None</td>
</tr>
<tr>
<td>2*</td>
<td>M/48</td>
<td>Lymph node metastasis</td>
<td>Not reported</td>
<td>2, 5, 7, 8, 9, 11p, 12, 14, 15, 21</td>
<td>18p</td>
</tr>
<tr>
<td>3*</td>
<td>M/29</td>
<td>Lymph nodes metastasis</td>
<td>Not reported</td>
<td>4, 5, 7q, 12, 13, 14, 18q</td>
<td>1p31qter, 10, 11q, 16, 22</td>
</tr>
<tr>
<td>4*</td>
<td>M/35</td>
<td>Lymph nodes metastasis</td>
<td>Not reported</td>
<td>4p15pter, 18q</td>
<td>1p31qter, 4q, 11q12q21, 16p, 17q, 19q</td>
</tr>
<tr>
<td>5*</td>
<td>F/40</td>
<td>Lymph nodes Metastasis</td>
<td>Not reported</td>
<td>7, 9, 14, 15, 17, 18</td>
<td>8p, 16p</td>
</tr>
<tr>
<td>6*</td>
<td>M/41</td>
<td>Lymph nodes metastasis</td>
<td>Not reported</td>
<td>4, 5, 7, 9, 12, 13, 17q11, 18</td>
<td>1, 16</td>
</tr>
<tr>
<td>7*</td>
<td>F/56</td>
<td>Lymph node metastasis</td>
<td>Not reported</td>
<td>17q</td>
<td>3, 1p</td>
</tr>
<tr>
<td>8*</td>
<td>F/50</td>
<td>Liver metastasis</td>
<td>Not reported</td>
<td>4, 5, 7, 9, 10q, 12, 13, 14, 17, 18q, 30</td>
<td>None</td>
</tr>
<tr>
<td>9*</td>
<td>M/61</td>
<td>Lymph nodes metastasis</td>
<td>Not reported</td>
<td>12, 13, 14, 17, 19, 20, 22</td>
<td>None</td>
</tr>
</tbody>
</table>

* Cases described in Zhao et al. [1].
* Cases described in Speet et al. [14].
* Cases described in Terris et al. [15].
Table 4
Chromosomal regions and/or genes that could be involved in NF PET tumorigenesis or malignancy

<table>
<thead>
<tr>
<th>Reference</th>
<th>Gains</th>
<th>Losses</th>
<th>Candidate genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terris et al., 1999 [15]</td>
<td>4p15-pter (50%) and 7 (50%) gains, 17q11 gain (60%)</td>
<td>3p, 6q, 8q, 10q, 11pq in more than 60%</td>
<td>HER2/neu protooncogene</td>
</tr>
<tr>
<td>Speel et al., 1999 [13]</td>
<td>5q, 7q, 12q, 14q, 17q in more than 60%</td>
<td>6q21 loss (50%) and 10p and 10q malignancy</td>
<td>MEC oncogene</td>
</tr>
<tr>
<td>Speel et al., 2001 [14]</td>
<td>4p (40%) and 4q (30%) gains and 4p (8%) and 4q (30%) gain malignancy associated (in NF PET and functioning PET)</td>
<td>High frequency of LOH in 6q and 11</td>
<td></td>
</tr>
<tr>
<td>Zhao et al., 2001 [1]</td>
<td></td>
<td>22q12.1 and 22q12.3 loss associated to malignancy</td>
<td>(in NF and in functioning PETs)</td>
</tr>
<tr>
<td>Rigard et al., 2001 [5]</td>
<td></td>
<td></td>
<td>High frequency of LOH in 6q, 11, 20q and 21</td>
</tr>
<tr>
<td>Wild et al., 2002 [6]</td>
<td></td>
<td></td>
<td>MEN1 mutations found in 27%</td>
</tr>
</tbody>
</table>

in ovarian carcinoma cell lines and primary tumors, as well as in pancreatic tumors of both non-endocrine and endocrine origin [25].

The 8q24.3 region was gained and highly amplified in two malignant cases. The PRL3 gene maps at this locus (MIM no. 616449), and according to Saha et al. (2001) [26], the PRL3 gene is important for metastasis in colorectal cancer. The KCNK9 gene (MIM no. 605874) has also been mapped in this region, and its amplification/overexpression plays an important role in human breast cancer [27]. These observations confirm that the 8q24.3 region is associated with tumor aggressiveness and malignancy in different tumor types.

In conclusion, our data support the hypothesis that the progression of PET toward malignant behavior requires an accumulation of genetic changes [1,6,13,14], and it seems likely that genes guarding chromosomal stability are involved in PET progression [6]. To date, CGH has been applied to only a limited number of investigations involving NF-PET. Our study focuses attention on the relevance of 11p13, 6q25, 6q27, 19q13.1, and 8q24.3 gains and 22q13.3 loss in the development and/or malignancy of this tumor type and thus contributes significantly to the accumulation of pertinent data.

Acknowledgments

We would like to thank Chris Tyler-Smith for critical reading and comments and Natalia Mancino for technical assistance in the preparation of the manuscript. This study was supported by Associazione Italiana per la Ricerca sul Cancro (Milan, Italy), Ministero dell’Istruzione, dell’Università e della Ricerca and Consiglio Nazionale delle Ricerche (“Diagnostica molecolare in oncologia”), and Fondazione Cariverona (Bando 2001).

References

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8. APPENDIX 2
ITALIAN XLMR BANK forms

Private data

First Name
Last Name
Date of Birth
Place of Birth
Residence City

Family History

-Familial case  -Sporadic case  -Likely familial  -Unknown

Clinical data of relatives: (text)

Anamnestic Form

Clinical History:
I.Q.:  
  value (1-100) =  
  age (1-100) =  
  scale (choose WPPSI, WISC-R, WAIS, Leiter, Griffith, Denever, Bailey, other) =

Adaptive Behaviour (Vineland Scale):  
  description (text):  
    -socialization : age (1-100) = age equivalent (1-100) =  
    -daily skills : age (1-100) = age equivalent (1-100) =  
    -communication : age (1-100) = age equivalent (1-100) =

Behaviour:  
  Description:  
  Age (1-100):  
  Scale (choose: CBCL, CARS, ABC, DBC, other):  
  Conclusion (choose: normal, autism, ADHD, PDD):

Other Examinations:  

EEG:
MRI/TAC:

Karyotype:
   Banding Resolution (choose: 350-500, 500-850, 850-1250, other) =
   Result =

FRAXA
   Test=
   Result=

Subtelomeric rearrangements:
   Technique:
   Result:

-------------------------------------

Clinical Data

Dysmorphic Examination:

Physical Examination:

Neurologic Examination:

-------------------------------------

Privacy

(physician who has the patient consent form; insert reference person, unit name, full address, phone number, e-mail)

Please attach the image of Pedigree as .jpeg File (please insert generation and individual number in the pedigree)

Please attach image of MRI and eventually of other examinations

Please attach the photos and movie of patient
9. APPENDIX 3
Subject: microarray scanner AQuire Genetix.

Dear Genetix Limited,

I’m writing to you about the performance of the microarray scanner AQuire (Genetix Limited) for the microarray based CGH application.

During 2004, the Medical Genetics Unit of the University of Siena approached the Italian distributor of Genetix products (Advanced Biotech Italia SRL) to purchase the equipment to specifically perform array-based comparative genomic hybridization. In particular, we received an offer to purchase the AQuire scanner at the cost of 15495,00 euros. The ABI agents have described the scanner AQuire as a suitable instrument for array-based Comparative Genomic Hybridization experiments without limitations linked to the use of different types slides (as also described in the Genetix website: http://www.genetix.com/instr/aquire.asp). Consequently, we decided to purchase the instrument.

On July 2004 we ordered the scanner AQuire (cat # X2840) and the spotter QArray mini (cat # X2700) to ABI SRL, for a total amount of 78922,40 euros + IVA offer # 130704/C 22/07/2004. Both instruments were installed and regularly tested on September 2004 (see attached document 1). The invoicing of the instruments was divided in several payments because it was executed on different funds. At present, we have effected all payments and remains only invoice#121/S, 24/05/2005, total amount 10750,00 euros.

Since September 2004 two researchers of my unit are full-time employed in the set up of the array-CGH protocol using BAC arrays specific for the X-chromosome and commercial whole-genome BAC slides. During this time they have improved their knowledge on the array-CGH techniques and protocols, acquiring the experience necessary to evaluate the quality of the experiments and the performances of different instruments. The array CGH protocols we are currently using have been validated by the slides producers.

I’m very sorry to inform you that the technical features of the instrument do not really fit the requirements necessary for the array-CGH application. The same slides acquired in a first moment with the AQuire scanner (Genetix) and then with a different scanner give very different results. The quality difference is so high that experiments acquired with AQuire scanner do not allow to obtain the final data.

In order to demonstrate what we sustain, we attach to this letter an illustrative array-CGH experiment (Fig. 1, 2, 3). Figure 1 shows an array-CGH experiment example, in which it is possible to view, that in the images acquired with the AQuire scanner the definition of the spots and the fluorescence intensities of both CY3 and CY5 dyes are extremely reduced with respect to the Affymetrix images (Affymetrix 428 Scanner, Affymetrix S. Clara, CA, USA). Moreover, the increase of laser emission intensities resulted in a pronounced background raising that has further reduced the quality of images. In the course of this year, we have tried to acquire some X-chromosome specific slides and whole-genome slides
deriving from different experiments and we repeatedly obtained very poor results. The reduced quality of images has an highly negative impact on the subsequent analysis and compromises the final results. In Fig. 2 and Fig. 3 we show the opposing results obtained analysing one experiment acquired with both scanners with the same method (GenePix Pro 5.0v analysis and normalization with the software of the University of Nijmegen). The analysis of the image acquired with Genetix AQuire Scanner does not permit to perform the final result (Fig. 2), while a such result on the contrary is obtained analysing the acquired image with Affymetrix 428 Scanner (Fig. 3).

From the above described data, we conclude that the AQuire scanner is not a suitable resource for array CGH technology contrary to what is specified on the Genetix website.

Since this has resulted in an economic and scientific damage for our laboratory (loss of expensive reagents and loss of time of 2 operators working at the set up for more than 10 months) we ask a compensation from damages to Genetix.

We request as damages refund to be exempted from the payment of the last invoice (invoice#121/S, 24/05/2005, total amount 10750,00 euros) and of the invoice # 274/S (17/11/2005, total amount 9936,00 euros) related to the order of the pins for the QArray mini spotter.

It remains understood that the scanner may be returned in any time.

Looking forward to hearing from you

Best regards

Alessandra Renieri

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Italy
tel: +39 0577 233303
fax: +39 0577 233325
Fig 1
Curriculum Vitae

Name: Chiara

Last name: Pescucci

Date of Birth: 05/02/1974

Place of Birth: Florence, Italy

Study:

February 2000: Degree in Biological Science at the University of Florence, Italy

Professional experience:


March 2001 – November 2001: worked in the “National Centre for Rare Diseases” at the National Institute of Health (Rome), under the supervision of Dr. Domenica Taruscio.

December 2001 – today: worked as Ph.D student in the Research Project “Identification and characterization of chromosomal rearrangements in patients with complex phenotypes” under the supervision of prof. Alessandra Renieri, Medical Genetics, University of Siena.
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