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Oligonucleotide Array Comparative Genomic Hybridization Profiling of Neuroblastoma Tumours

PAOLA SCARUFFI[1], STEFANO MORETTI[2], SIMONA COCO[1], KATIA MAZZOCCO[3], RAFFAELLA DEFFERRARI[3], DOMENICO ALBINO[3], STEFANO BONASSI[2] and GIAN PAOLO TONINI[1]

[1]Unit of Translational Paediatric Oncology and [2]Molecular Epidemiology, National Institute for Cancer Research (IST), Genoa; [3]Laboratory of Italian Neuroblastoma Foundation, Advanced Biotechnology Centre, Genoa, Italy

Abstract. *Neuroblastoma (NB) is one of the most common paediatric solid tumours and displays a broad variety of genomic alterations. Recently, array comparative genomic hybridization (aCGH) has emerged as a novel technology enabling high-resolution detection of DNA copy number aberrations. We have previously optimized a custom cDNA-array to detect MYCN gain and chromosome 1p36 loss, two molecular markers of tumour aggressiveness in NB. In spite of the power of this technique, the production of cDNA arrays is time-consuming and expensive. In the present study, we report a printed 55-mer oligonucleotide aCGH with the aim of increasing the resolution and the sensitivity of our platform. The oligonucleotide probes, designed and validated for expression profiling, reproducibly assessed amplifications, even when using whole genomes as targets. On the contrary, this microarray platform seems to offer little accuracy in measuring genomic single-copy deletions. Therefore, an oligo library specifically designed for aCGH should improve the performance of oligonucleotide aCGH in accurately mapping unbalanced chromosomal abnormalities.*

Neuroblastoma (NB) is one of the most common paediatric solid tumours and is responsible for approximately 15% of paediatric cancer death. The disease is characterized by a heterogeneous clinical behaviour, including maturation, spontaneous regression and rapid malignant progression (1). Many important factors such as stage, age at diagnosis and ploidy have been identified as being associated with the biological and clinical heterogeneity of NB tumours. Complex patterns of genetic abnormalities, that contribute to the malignant phenotype, have been described (2, 3). Amplification of the *MYCN* oncogene occurs in 20-25% of primary NBs and is a reliable marker of aggressive clinical behaviour. Several structural and numerical non-random chromosome abnormalities are frequently detected in NB and their role in the disease is being actively investigated. For example, deletions of 1p36 and 11q23, both associated with a worse prognosis, have been identified in up to one-third of primary tumours (4, 5). The unbalanced gain of 17q material is also associated with an adverse prognosis (6). Other regional amplifications and deletions have been identified in NB but are not as well characterized (7).

Metaphase comparative genomic hybridization (mCGH) is a molecular cytogenetic technique that has substantially increased the genome-wide information on unbalanced chromosomal changes compared to conventional cytogenetics and fluorescence *in situ* hybridization (FISH) analysis (8). According to the literature, mCGH analyses of NB detected many novel and non-random genomic alterations, mostly consisting of *MYCN* amplification, 17q gain and deletions at the 1p36, 3p, 4p, 9p, 11q and 14q regions (9-19). However, this technique is hampered by a low resolution of 5 -10 Mb (8, 20) so that small genetic changes may be not identified. These limits are overcome using array CGH (aCGH), a novel technology for genome wide high-resolution detection of DNA copy number aberrations. Instead of metaphases, aCGH uses arrayed sequences of DNA bound to glass slides and probed with the genomes of interest (21-26). Platforms with bacterial artificial chromosomes (BACs), phage artificial chromosomes (PACs) and cosmids as probes yield a resolution of 1-1.5 Mb (23), whereas cDNAs yield an average resolution of 267 kb (27, 28). Several studies have documented the utility of BAC, PAC or cDNA-based microarrays for the CGH profiling of NB (25, 29-33).

We had previously optimized a custom cDNA-array to detect *MYCN* gain and chromosome 1p36 loss, two determinants of tumour aggressiveness in NB (34). This analysis of primary NB tumour genomes demonstrated that the cDNA aCGH platform is sufficiently robust to detect both

Correspondence to: Dr. Paola Scaruffi, Unit of Translational Paediatric Oncology, National Institute for Cancer Research (IST), L.go R. Benzi, 10, 16132 - Genoa, Italy. Tel/Fax: +39-010-5737463, e-mail: paola.scaruffi@istge.it

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1p36 chromosome loss and *MYCN* amplification, with a sensitivity and specificity of ~67% and 90%, respectively. In spite of the power of this technique, the production of cDNA arrays is time-consuming and expensive. The efforts to improve our approach took two directions: i) design of a new NB-specific microarray containing 55-base long nucleotide probes, in order to increase the sensitivity of our aCGH in detecting low levels of gene copy number gains and losses; ii) improvement of the resolution of this method, including probes mapping at various chromosome regions, namely 1p13.2-p36.33, 2p13.3-p24.3, 2q36.1, 7q21.11-q34, 9p21.3-p24.2, 11q12.2-q23.2, 12q14.1-q24.31, 14q24.2-q33 and 17q11.2-q25.3 and baseline oligos mapped at non NB-related regions.

It was demonstrated that the oligonucleotide aCGH platform improved the detection of DNA gains, but it was less accurate in measuring genomic single-copy detection. It is foreseen that a specifically designed aCGH oligonucleotide microarray would better map unbalanced chromosomal abnormalities in a global and detailed manner.

Materials and Methods

Tumour sample collection. Tumour samples were collected from 15 patients of the Department of Haematology and Oncology of the G. Gaslini Children's Hospital of Genoa, Italy. Samples were collected at the onset of disease with the approval of the Ethical Committee of the Institute and after obtaining the parents' written consent. Each surgically-resected tumour was processed by pathologists according to the SIOP Europe Neuroblastoma Pathology, Biology and Bone Marrow Group guidelines (35) and immediately stored at –80˚C. Histological classification was performed according to Shimada *et al.* (36) and the cell content was evaluated for each sample. Only tumours containing more than 80% of malignant cells were included in the study. The DNA content was analysed by cytoflourimetric assay on formalin-fixed, paraffin-embedded samples, carried out under a cytofluorimeter (FACScan, Becton Dickinson, NJ, USA) on at least 20,000 events. The results were analysed by means of ModFit LTTM software (Verity Software House, USA). The patients' peripheral blood cells were collected with the aim of providing normal reference DNA for the assessment of gene copy changes in the tumour samples.

Double-colour FISH on tumour interphase nuclei and LOH-PCR for chromosome 1p36. Chromosome 1p36 deletion was assessed by double-colour FISH on interphase nuclei of cell tumour touch preparations. The P1-79 (locus D1Z2 at 1pter) and QC (chromosome 1 centromere) probes were labelled with biotin-16-dUTP and digoxigenin-11-dUTP, respectively. *MYCN* amplification was detected using the *MYCN* and D2Z probes (chromosome 2 centromere) (Appligene Oncor). LOH was studied by a PCR-based method using primer sets for D1S80 and D1S76 loci mapping to subtelomeric region of chromosome 1p36. FISH results for chromosome 1p36 and *MYCN* and LOH for D1S76 and D1S80 have been reported according to Ambros *et al.* (37).

Array comparative genomic hybridization (aCGH). Total genomic DNA (gDNA) was extracted from the tumour samples and normal peripheral blood lymphocytes of NB patients, according to Sambrook *et al.* (38). In order to optimize the protocol, 5 μg of gDNA of each sample were treated with DNAse to obtain fragments smaller than 500 bp. After purification with the QIAquick-PCR purification kit (Qiagen GmbH, Hilden, Germany), fragmented gDNA was labelled by direct enzymatic incorporation of fluorescent tags. The gDNA was labelled with Cy3-dCTP or Cy5-dCTP (Amersham Biosciences, Piscataway, NJ, USA) using the Random-Primed Bioprime DNA Labelling kit (Invitrogen Life Technologies, Carlsbad, CA, USA). Briefly a 50-μl reaction contained dATP, dGTP and dTTP (120 μM each), dCTP (60 μM) and Cy3-dCTP (60 μM) or Cy5-dCTP (60 μM). After incubation with Klenow Fragment (40 units) at 37˚C for 2 h, the reaction was stopped with 0.5 M EDTA, pH 8.0. The average size of the fragmented labelled target was between 50 and 200 bp. Unincorporated nucleotides were removed on a Sephadex G50 microspin column (Amersham Biosciences), according to the manufacturer's protocol. The pooled Cy3- and Cy5-labelled DNA targets were mixed with 30 μg of human Cot-1 DNA (Invitrogen Life Technologies) and 100 μg of yeast tRNA (Invitrogen Life Technologies), 20 mg of poly(dA-dT) (Sigma) and ethanol-precipitated. The solution was rinsed twice in 70% cold ethanol and air-dried.

A total of 205 probes, that are 5'-MMT modified 55-base long nucleotide oligonucleotides (MWG Biotech, Ebersberg, Germany), were used. The oligonucleotides were selected at chromosome regions known as frequently deleted (1p13.2-p36.33, 9p21.3-p24.2, 11q12.2-q23.2, 14q24.2-q33) and gained (2p13.3-p24.3, 2q36.1, 7q21.11-q34, 12q14.1-q24.31, 17q11.2-q25.3) in NB cells. The arrays also contained baseline oligonucleotide mapping at the 1q21.2-q23.2, 2q13-q34, 3q22.3-q25.1, 5p13.1-5q34, 6p22.1-6p25.1, 7p11.2-p22.1, 8p12-q23.1, 9q22.31-q31.3, 11p15.1-p15.4, 12p13.31, 14p11.2, 15q21.2 and 17p11.2-p13.3 regions. These baseline features have been considered as referere genes. Furthermore, *Arabidopsis thaliana genes (RCA, rbcL, Cab)* (Stratagene, Hogehilweg, The Netherlands) were used as the control for unspecific hybridization. Our microarray yielded an average resolution of 1 Mb within each chromosome region. The oligonucleotides were suspended in 3x SSC in order to give 100 pg/spot of probe and they were spotted by the SpotArray 24 (Perkin-Elmer Precisely, Wellesley, MA, USA) on CreativeChip® Oligo slides (ELIPSA GmbH, Germany). The features of the microarray were arranged on four subgrids. In each subgrid all probes were placed in quintuplicate, for a total of 1044 spots. *Arabidopsis thaliana* DNA was spotted in each subgrid following a squared design. This design allowed control over the independence of technical replicates and provided a suitable measure of non-specific hybridization within each experimental unit. A post-printing quality control was performed by using the "red reflect" function of ScanArray™ 4000XL (Perkin-Elmer Precisely). Three identical sub-arrays were arranged on each glass slide so that three replicates could be performed for each sample, except one sample which was replicated only twice.

The printed slides were incubated at 120˚C for 30 min. After coupling, the DNA was cross-linked to the slide surface by exposure to 0.30 J/cm^2 UV light in the UV CrossLinker Bio-Link® BLX-312 (Eppendorf, Hamburg, Germany). The slides were rinsed in 0.1% SDS for 30 sec and then in distilled water for 15 sec.

The slides were pre-hybridized at 42˚C for 4 h, with 15 μl of hybridization mix (50% formamide, 5x SSC, 5x Denhardt's reagent, 0.5% SDS, 5 mM potassium phosphate, at pH 7.2) containing 30 μg of salmon sperm DNA (Eppendorf). The slides

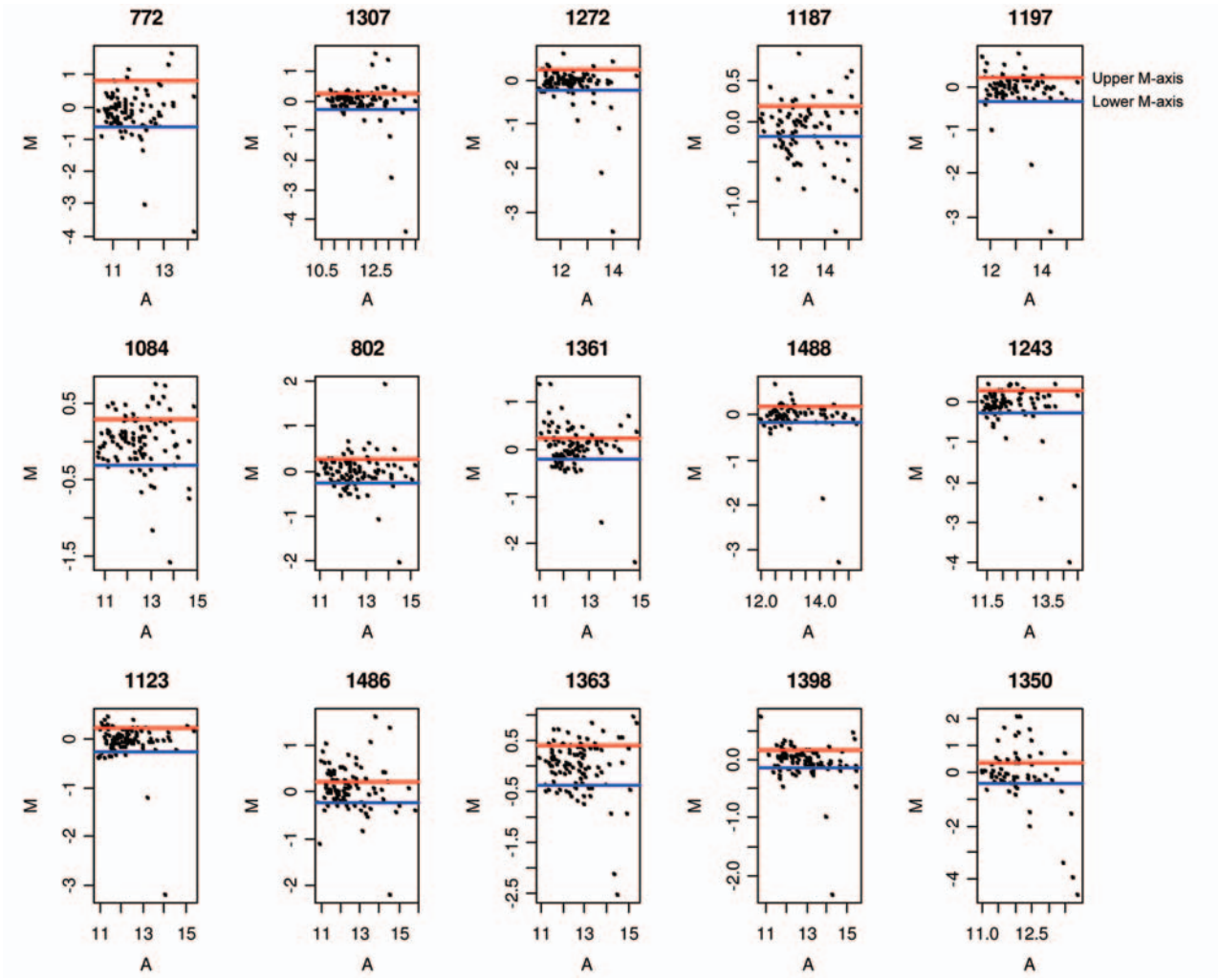

Figure 1. *MA-plots of the average log-ratios (M-values) for each NB sample. Being R and G, the normalized and scaled red and green intensities for each spot, respectively, the expression log-ratio corresponding to a spot is* $M=log_2R-log_2G$*, whereas the log-intensity of a spot is defined as* $A=(log_2R+log_2G)/2$*, a measure of the overall brightness of the spot. Each point above the upper red line corresponds to a gain, whereas each point below the lower blue line corresponds to a loss. For each sample, upper red and lower blue M-axis intercepts correspond to the mean* c^+_{ji} *and the mean* c^-_{ji}*, respectively.*

were hybridized in hybridization cassettes (Telechem International, Inc., Sunnyvale, CA, USA) with a mixture containing target DNAs, 30 μg of human cot-1 DNA and 100 μg of yeast tRNA (denatured by boiling for 5 min and pre-annealed at 37˚C for 2 hrs) and submerged in a 42˚C-waterbath for 16-20 h. After hybridization, the slides were gently washed with 2x SSC and 0.1% SDS at 42˚C for 3 min and with 0.2x SSC and 0.1% SDS at room temperature for 5 min. Finally, the slides were washed at room temperature for 5 min with 0.2x SSC.

Spot fluorescence was measured by the ScanArray™4000XL scanning laser confocal fluorescence microscope (Perkin-Elmer Precisely). Quantification was performed by the fixed method (QuantArray® Analysis software, Perkin-Elmer Precisely), which constructs spot and background masks using spot and background inner/outer diameters. The mean signal intensity was calculated as the sum of the intensity values of each pixel inside the signal area divided by the total number of pixels in the signal region. The same procedure was applied for the background intensity, with the exception that only pixels outside the signal area were considered. Spots showing abnormal hybridization signals were not included in any further analysis.

Statistical methods. Spots with a signal-to-noise ratio lower than 2.1 were considered unreliable and were removed before the statistical analysis. Background-subtracted fluorescence log-ratios were normalized within each array by using print-tip loss normalization (39) available in the Bioconductor *limma* package (40, 41). Print-tip loss normalization was applied in order to correct the expression log-ratios both for sub-array spatial variation and for intensity-based trends. In practice, this is done by subtracting from each expression log-ratio the corresponding value of the tip-group loess curve, where the loess curve in each tip-group is constructed by performing a series of local regressions, one local regression for each point in the MA-plot

(42). Only spots corresponding to the reference genes have been used in the construction of the tip-group loess curve. In order to make all technical replicates comparable, expression log-ratios of each replicate was scaled so as to have the same median-absolute-deviation (MAD) across all the arrays in each group of replicates. The minimum requirement was the homogeneity of MAD's within replicates of the same sample. To scale the expression log-ratios, the algorithm proposed by Yang *et al.* (43) was adopted, in the implemented version by Smyth (44) available in the *limma* package. A threshold method for selecting genes with extreme log-ratios was then applied to automatically classify gene gains or losses. In each array, all the normalized expression log-ratios corresponding to the 67 reference genes were plotted to a normal quantile-quantile (QQ) plot to establish the threshold bounds for the gain/loss assessment. Based on the observation of the proportions of log-ratios which lay on the line which passes through the first and third quartiles in all the QQ plots, for each replicate *j* of each sample *i*, the two thresholds, c^{+}_{ji} and c^{-}_{ji}, were computed such that $c^{+}_{ji}=mean_{ji}+sd_{ji}$, where $mean_{ij}$ and sd_{ij} are the mean and the standard deviation of control genes log-ratio distributions corresponding to the *j* replicate of the *i* sample, respectively. Then, for each sample *i*, those genes whose mean log-ratio according to the replicates of sample *i* was greater than the mean c^{+}_{ji} were scored as gain. Conversely, those genes whose mean log-ratio on the replicates of sample *i* was lower than the mean c^{-}_{ji} were scored as loss.

Results

In this study, the ability of long oligonucleotide (55-mer) aCGH to detect and to map regions of gain and loss throughout the genome was investigated. To first test the quality of the assay and the intrinsic variability of the method, DNA obtained from pooled genomic DNAs extracted from peripheral blood lymphocytes of NB patients was used. Hybridizations with normal DNA on both channels were performed using the same batch of microarrays, with identical labelling and hybridization conditions applied to the tumour analyses. Three replicate aCGH experiments demonstrated good reproducibility and, as expected, no gene copy number changes were found (data not shown). Next, the copy number variations in 15 primary NB tumours were measured. Figure 1 shows the MA-plots of the normalized average log-ratios (M-values) for each NB sample. Upper and lower M-axis intercepts correspond to the mean c^{+}_{ji} and the mean c^{-}_{ji}, respectively. The red line corresponds to the threshold to be reached for a gene to be scored as amplified. Conversely, each point below the lower line represents a loss.

In these microarrays the percentage of non-hybridizing oligonucleotides was high ($\sim$40%). Because of low signal-to-noise ratios, hybridization to some oligonucleotides was inadequate to allow precise analysis of that portion of the genome and to detect gains and losses with sufficient statistical certainty.

To assess the accuracy of the imbalances detected by aCGH analysis, the aCGH results were compared to conventional methods of copy number determination at selected loci. The aCGH results were verified with double-colour FISH on interphase nuclei and genotyping by PCR specific for the two major prognostic markers for NB (*MYCN* locus and chromosome 1p36 region) (Table I). According to the FISH analysis, *MYCN* amplification and 1p36 chromosome loss were observed in seven samples (#772, #802, #1123, #1243, #1307, #1363, #1486), while sample #1488 showed only 1p36 loss. Chromosome 1p FISH data were not available for sample #1187, though no evidence of 1p36 LOH was detected by PCR. There was complete concordance between the aCGH results and the *MYCN* locus genomic amplification detected by FISH. Altogether, genes mapping to chromosome band 2p24 were observed to be co-amplified with *MYCN*, including *DDX1* and neuroblastoma amplified gene (*NAG*). This implies that in all seven *MYCN*-amplified samples the *MYCN* amplicon is a $\sim$2 Mb region (www.ensembl.org).

Furthermore, the sensitivity and the specificity of our aCGH platform in detecting gains and losses were estimated. For this purpose, FISH for the *MYCN* gene and chromosome 1p36 was used as a reference method to calculate the correctly identified ratios of gain/loss and of no gain/no loss. Table II shows that the mean sensitivity of aCGH in detecting a loss of the 1p36 chromosome region based on the 14 genes selected was 8.7%, with a specificity of 78.4%. Looking at each oligonucleotide mapping at the 1p36 chromosome region, *p73* showed the highest sensitivity and four probes (*TNFRSF1B*, *HBACH*, *KIAAA0444* and *CDC2L1*) were never classified as false-positive (specificity 100%). *MYCN* amplification was correctly recognized by aCGH in all samples reported as positive by FISH analysis. Therefore, the sensitivity of aCGH in detecting *MYCN* amplification was found to be 100% and the specificity 87.5%, due to one false-positive value (sample #1361).

Discussion

A number of reports of conventional chromosome-based CGH have described a wide variation of numerical aberrations in NB tumours (8-11, 13-19). However, the relatively low resolution of mCGH means that further experiments are necessary to provide detailed positional mapping of gained/lost sequences of interest. To overcome this limit, several authors performed aCGH on BACs, PACs, cosmids or cDNAs to detect unbalanced chromosomal aberrations with high resolution (25, 30, 31, 33, 35).

Efforts have been made to improve the resolution and the sensitivity of our previous cDNA-based NB-specific platform (34). We have designed a 55-mer oligonucleotide microarray that covers several regions containing genes known to be involved in NB progression.

It is noteworthy that the eligibility of the NB samples for this study was carefully checked, in order to avoid tumour/normal hybridization ratio bias due to tumour heterogeneity, the

Table I. *Evaluation of MYCN gene status and chromosome 1p36 loss with FISH in 15 NB samples.*

	FISH	
Sample #	*MYCN* gain	1p36 loss
772	Yes	Del
802	Yes	Del
1084	No	No
1123	Yes	Del
1187	No	No LOH[a]
1197	No	n.d.
1243	Yes	Del
1272	No	No
1307	Yes	Del
1350	No	No
1361	No	No
1363	Yes	Del
1398	No	No
1486	Yes	Del
1488	No	Del

[a]presence of PCR-based LOH; n.d.=not determined; Del=deletion.

Table II. *Sensitivity and specificity of MYCN gain and chromosome 1p36 loss by aCGH (standard classification based on FISH/LOH PCR).*

		True positives		True negatives	
		N*	%	N*	%
Gain					
	MYCN	7/7	100	7/8	87.5
Loss					
	Chromosome 1p				
	HSPG2	1/7	14	5/8	62
	ID3	0/7	0	3/4	75
	CASP9	0/7	0	4/7	57
	NBL1	0/7	0	7/8	87
	TNFRSF1B	0/7	0	8/8	100
	UBE4B	0/7	0	5/7	71
	HBACH	0/7	0	8/8	100
	ICMT	1/7	14	7/8	87
	KCNAB2	0/7	0	4/5	80
	KIAA0444	1/5	20	8/8	100
	NPHP4	0/7	0	6/8	75
	RPL22	0/7	0	7/8	87
	P73	4/7	57	1/6	17
	CDC2L1	1/6	17	6/6	100

*N: for each probe only spots with a signal-to-noise ratio higher than 2.1 were considered.

presence of Schwann stromal cells, granulation, fibrous and lymphoid tissues in the sample. Histology was evaluated before DNA purification and only diploid DNA specimens containing more than 80% of malignant cells were included in the analysis.

MYCN amplification remains a powerful predictor of poor survival probability in NB patients (45). According to the European Neuroblastoma Quality Assessment Group guidelines, Southern blot and PCR are not recommended for use as the only methods for *MYCN* evaluation without FISH analysis (35, 37). *MYCN* heterogeneous amplifications or gains cannot be reliably detected by Southern blot and PCR methods, unlike FISH, which is done at the single cell level. Our results showed that the allelic status of 2p24 (*MYCN*) was detected by aCGH, in complete concordance with the data obtained by FISH technique.

MYCN amplification usually involves a large and variable number of DNA sequences flanking the *MYCN* region at chromosome 2p24. Our aCGH platform was able to characterize both the extent and the complexity of the amplified sequences associated with the *MYCN* amplicons that have been difficult to identify by cytogenetic methods. It was observed that genes mapping to chromosome band 2p24 were co-amplified with *MYCN*, including *DDX1* (*DEAD/H-BOX1*) and the neuroblastoma amplified gene (NAG). *NAG* has previously been observed to be part of the *MYCN* amplicon, although its function is still unknown (46). The oncogene *DDX1* belongs to a family of genes that encode DEAD box proteins, putative ATP-dependent RNA helicases (47). Amplification of *DDX1* was observed to be associated with *MYCN* amplification in human NB cell lines and in 50-70% of primary tumours (48-53). Therefore, the utility of this oligonucleotide aCGH for characterizing gene amplifications was demonstrated.

Deletions of the short arm of chromosome 1 are common chromosome alterations of NB cell lines and have been found in 19% to 36% of primary tumours (54-57). There is a correlation between 1p loss and high-risk features such as age, diagnosis over one year, metastatic disease and *MYCN* amplification. Thus, chromosome 1p analysis may identify patients who are more likely to suffer disease relapse (55, 56).

Previous reports from Takeda *et al.* (58) and from our group (59) showed that interstitial and large deletions are detectable both in localized and disseminated tumours. This is an important issue because it has been postulated that tumours with large deletions generally also have *MYCN* amplification and a poor probability of survival, whereas those with smaller deletions are more likely to have a single copy of *MYCN* and a favourable clinical outcome (58). The oligonucleotide-based aCGH approach has the advantage of offer the possibility of detecting 1p loss at several loci in a single assay. In order to provide an extensive coverage of the 1p chromosome, thirty-four oligonucleotides were printed, spanning from 1p36.33 to 1p13.2 region. This also implies that losses are mapped by their gene position and that the analysis can directly provide a list of candidate NB-associated genes within the region of interest.

One long-standing concern has been whether the high complexity of the full genome would undermine the accurate

reporting potential of short DNA probes on a microarray. First, two types of oligo-microarrays have shown their usefulness in detecting genomic alterations (60-62). One platform uses photoprinted custom-designed 70-mer, whereas another is a single-nucleotide polymorphism array. In both cases, a PCR-based genomic representation is required to reduce the complexity of input genomic DNA by 98% as a means to improve the hybridization kinetics (60-63). Recently, Brennan *et al.* (64) provided evidence of the reliable detection of single copy number alterations in full-complexity genomic DNA, using a commercially available 60-mer platform. Our study highlighted that the PCR enrichment step is not necessary to reduce the genome complexity before hybridization to the microarray. Direct genome labelling and hybridization eliminate prevailing concerns about the impact of PCR amplification biases on the result.

This study demonstrated that the 55-mer oligonucleotide probes, designed and validated for gene expression profiling, can reproducibly assess variable amplicons even using whole genomes as targets. On the contrary, these molecules are unreliable features for the detection of single-copy deletions with high sensitivity. Our findings imply that this microarray platform optimized for mRNA profiling achieves measurements of genomic DNA copy number changes with low accuracy. These probes, although unique in the transcriptome, could have homology with more than one site in the whole human genome. Moreover, in our experiments it was necessary to discard measurements on several clones because they did not provide valuable signals. These non-hybridizing oligos could be explained by the fact that they cross mRNA splice boundaries and, consequently, are not contiguous in the genome.

We believe that the performance of our oligonucleotide platform could be improved by using a dedicated oligo library specifically designed for aCGH. Such a genomic array may provide representations of both gene-specific and unique intragenic sequences. The oligos targeted to noncoding DNAs may represent important regulatory regions, including regulatory elements and microRNAs. Moreover, the commercial availability of oligonucleotide libraries specifically dedicated to aCGH will guarantee standardization and reproducibility, two important issues especially in diagnosis. Finally, this perspective obviates the need for in-house microarray printing facilities and provides increased access to the scientific community. Each approach has its own advantages and disadvantages, with commercial sources undoubtedly costing more in comparison to in-house-printed arrays. In the future, the balance between costs and the robustness of the data will be assessed in order to choose the most suitable platform for each specific aCGH application.

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