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Comparative study of MLPA-FISH to determine DNA copy number alterations in neuroblastic tumors

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Summary. Neuroblastoma tumor cells show complex combinations of genetic aberrations, and to date many different methods have been used for their detection. To apply genome-wide techniques, such as Multiplex Ligation–dependent Probe Amplification (MLPA), in routine diagnosis their validation is appropriate and necessary. DNA copy number alterations in 129 cases of neuroblastic tumors were detected using MPLA, and the results validated by Fluorescence *In Situ* Hybridization (FISH) (*MYCN* gene, 1p36, 11q and 17q). Kappa index values showed very good concordance between the two techniques in detecting homogeneous *MYCN* amplification (1); 11q deletion (0.908) and 17q gain (0.922). The validation results showed that MLPA is a highly efficient technique for diagnosis based on the genetic aberrations in relevant regions in neuroblastoma, showing a high concordance with FISH.

Key words: Neuroblastoma, Genetic alterations, FISH, MLPA, Validation

Introduction

Neuroblastic tumors (NB) are the most common extra-cranial solid neoplasms in childhood. They show a diverse behavior, ranging from rapid malignant progression to spontaneous regression, and both prognosis and response to therapy can vary widely (Castel et al., 2001). Furthermore, despite extensive study, misclassification of patients still occurs, and the accurate prediction of the clinical course remains a challenge in NB research.

The International Neuroblastoma Risk Group (INRG) classification system was developed to establish a consensus approach for pre-treatment risk stratification, and new strategies for risk-based therapies are now being developed. Stage, age, histological grade of tumor differentiation, the *MYCN* oncogene and chromosome 11q status, and DNA ploidy are presently the most statistically significant relevant factors (Cohn et al., 2009; Monclair et al., 2009). However, experience suggests that such risk classification will still be suboptimal for a substantial number of patients. It is anticipated that any future INRG classification system will rely on the genetic profile of NB tumors, rather than on the presence or absence of individual genetic alterations.

Recently, Janoueix-Lerosey et al. (2009) showed that NB tumors presenting exclusively whole chromosome copy number variations were associated with excellent survival, and tumors with any type of segmental chromosome alterations characterized patients with a high risk of relapse. Different losses of heterozygosity have been reported for other chromosomal regions in NB, such as 3p, 4p, 9p, and 12p (Caron et al., 1996; Schleiermacher et al., 2007). Studies suggest that low-risk tumors may be best defined by the absence of *MYCN* amplification and of any other structural genetic abnormalities (Vandesompele et al., 2005; Schleiermacher et al., 2007). The analysis of the overall genomic pattern is essential to predict the prognosis in NB patients and new markers should be included in future treatment stratification.

To date, many different methods have been used for the detection of deletions and gains, including chromosome analysis (Betts et al., 2005), Southern blot (Sartelet et al., 2002), Fluorescence *In Situ* Hybridization (FISH) (Wan et al., 2004), Metaphasic-Comparative Genomic Hybridization (mCGH) (Schleiermacher et al., 2007) and real-time quantitative PCR (Boensch et al., 2005). However, these methods all have some limitations. More recently, array-CGH (aCGH) and Single Nucleotide Polymorphism (SNP) based platforms

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have been applied in order to detect genomic alterations in NB (Chen et al., 2004; George et al., 2005; Selzer et al., 2005; Carr et al., 2007; Scaruffi et al., 2007; Caren et al., 2008; Janoueix-Lerosey et al., 2009). These methods improve resolution and sensitivity; they are very robust techniques for the detection of small extracopies or microdeletions (cryptic) chromosome rearrangements, but are labour intensive and require expensive equipment. Multiplex Ligation–dependent Probe Amplification (MLPA) is a new and fast method first described in 2002 (Schouten et al., 2002). It is PCR based and allows the copy number status of up to 45 DNA sequences to be determined simultaneously. Several studies have been published, the majority using commercial MLPA assays to detect gene deletions and duplications in different disorders (Erlandson et al., 2003; Hogervorst et al., 2003; Nakagawa et al., 2003; Jeuken et al., 2006; Alibakhshi et al., 2008). The NB-specific MLPA kits allow the detection of DNA copy number variations of chromosomal regions of highest interest in NB. Our group has previously used different genomic techniques (FISH, mCGH, aCGH and MLPA) to detect chromosomal and gene alterations in patients with NB, proposing the FISH technique to visualize isolated cells and detect alterations present in a low percentage of tumor cells (Villamon et al., 2008). The INRG Biology Committee recommends using array-based methods, MLPA or similar techniques in the future to detect segmental chromosome aberrations. These techniques are currently undergoing validation (Ambros et al., 2009).

In this study MLPA was used to detect chromosomal and gene copy number alterations in 129 patients with NB, validating the results by FISH, with the aim of using MLPA in future routine diagnosis in NB.

Materials and methods

Samples

Samples were referred to the Spanish Reference Centre for NB Biological and Pathological studies at the time of diagnosis. Touch preparations and paraffin slides were stained with hematoxylin-eosin (HE) and examined by the pathologist to evaluate the amount of neuroblastic cells and histopathologically categorized according to the International Neuroblastoma Pathology Classification (INPC).

Mutiple ligation-dependent probe amplification (MLPA)

Tumors included for DNA extraction had a minimum of 40% neuroblastic cells. DNA was extracted using a phenol/chloroform/isoamyl alcohol extraction after proteinase K treatment. MLPA was performed using the SALSA MLPA Kit P251/P252/P253 (version A1), developed by MRC-Holland in co-operation with SIOPEN (SIOP Europe Neuroblastoma) (www.mrc-holland.com). This kit can be used to detect copy number changes of the 10 chromosomal regions that frequently show these alterations in NB. The SALSA MLPA P251 probemix contains probes for chromosomes 1, 3 and 11; P252 probemix for chromosomes 2 (*MYCN* region) and 17; and P253 probemix for chromosomes 4, 7, 9, 12 and 14. Furthermore, each panel includes control probes located in chromosomal regions rarely altered numerically in NB (reference chromosomes). MLPA was performed as described by the manufacturer with minor modifications. MLPA probes consist of two separate oligonucleotides, each containing one PCR primer sequence, which can ligate to each other. The ligation products were amplified by PCR using the common primer set with the 6-FAM label distributed by the supplier. The number of probe ligation products depends on the number of target sequences in the sample. Fragments were separated and quantified by electrophoresis on an ABI 3730 sequencer and the results analyzed using Genemapper software (Applied Biosystems). For data processing, at least three normal control DNA samples were included in each set of MLPA experiments. Reference DNA was isolated from the blood of healthy volunteers. Data analysis was performed with Coffalyser MLPA-DAT software (MRC-Holland, Amsterdam, The Netherlands) generating the normalized peak value or the so-called probe ratio. The threshold for detecting losses and gains in tumor samples was set at 0.75 and 1.25, respectively. A graphical visualization of the ratio values with Microsoft Excel was created. Signal intensity is visualized in the graphic representation of the MLPA results as height of the bars (Fig. 1).

Recently, chromosome aberrations analyzed by MLPA have been defined (Ambros et al., 2009). Normal status is considered as a balanced ratio between the majority of signals of both chromosome arms. Loss and gain are considered as an unbalanced ratio (low and high signal excess respectively) between the signals of the chromosomal region of interest (at least 2 adjacent probes) and the reference signals (at least 2 probes). A gain result could also correspond to a FISH deletion of the opposite chromosome arm and/or a FISH imbalance. We have defined the term ‘1p imbalance detected by MLPA’ for cases of 1q gain detected by MLPA in order to relate it with 1p imbalance shown by FISH. *MYCN* amplification is defined as unbalanced ratio (high signal excess) between the gene signals and all other probes located on the same chromosome. For the experimental system, the threshold between gain and amplification needs to be determined with the help of other techniques (for example Interphase-FISH). In our experience, amplification can be diagnosed if at least 2 adjacent probes of a gene show a ratio or grade ≥3.

Fluorescence In Situ Hybridization (FISH)

Touch preparations were used for FISH. *MYCN* copy number, 1p deletion, 11q deletion and 17q gain were investigated with commercial probes: *MYCN*

(2p24)/*LAF* (2q11); *ATM* (11q22) /SE 11; *MPO* (17q22) ISO 17q/*p53* (17p13), cocktail probes dual colour direct labelled (Kreatech, Biotechnology) and 1p36 (*D1Z2*)/centromere Chr.1 (Qbiogene). Assessment and interpretation of FISH results were performed according to previously published procedures (Ambros et al., 2003, 2009). A balanced ratio between the signal numbers of the chromosomal region of interest and the reference signals on the opposite arm of the chromosome is described as disomic (ratio 2:2) and aneusomic or whole extracopy chromosomes (i.e. ratios 3:3, 4:4, 5:5 etc.). An unbalanced ratio between the signal numbers of the chromosomal region of interest and the reference signals with only 1 signal of the chromosomal region of interest is considered deletion (i.e. ratios 2:1, 3:1, 4:1 etc.), and with more than 1 signal of the chromosomal region of interest is considered imbalance (i.e. ratios 3:2, 4:3, 5:3 etc.). In deleted cases a percentage of tumor cells can also include imbalance. No deleted cells are shown in imbalance cases. Gain is considered when signal numbers of the chromosomal region of interest exceed by up to 4 times the number of reference signals (i.e. ratios 2:3, 3:4, 3:5 etc.). Homogeneous amplification is considered when there is a more than a 4-fold increase in the *MYCN* signal number compared with the reference probe located on chromosome 2 and all tumor cells present *MYCN* amplification. Co-existence of amplified tumor cells beside non-amplified tumor cells in the same tumor is considered as heterogeneous amplification.

Statistical analysis

The kappa index was used to determine the concordance between FISH and MLPA results. The Kappa statistic assesses the extent to which models predict occurrence at a rate higher than expected by chance (Landis and Koch, 1977), and with 95% confidence intervals is good for assessing such results. A kappa statistic of 0 indicates the same level of agreement as expected by chance, 1 indicates perfect agreement. Cases with low quality DNA and inconclusive results were not included in the statistical analysis.

Results

Table 1 shows the number of cases with DNA copy number alterations by FISH and MLPA, the kappa index obtained in the detection of the different alterations and the concordance between both techniques.

Regarding 1p deletion, FISH detected 45 cases, of which 18 (14.1%) were not detected by MLPA. Concordance between both techniques was good, with a coincidence in 83.6% of the samples (kappa index: 0.610). For 1p imbalance detection, the two techniques coincided in 87.4% of the samples, the kappa index was poor (0.132), and may have arisen because only two of the ten cases with 1p imbalance detected by FISH were also detected by MLPA.

Considering *MYCN* amplification, (heterogeneous plus homogeneous *MYCN* amplification), concordance between both techniques was good (kappa index: 0.745). FISH detected heterogeneous *MYCN* amplification in 6 samples (4.7%), but these were not detected by MLPA. All cases with homogenous *MYCN* amplification detected by FISH (n=10) were detected by MLPA.

Regarding 2p gain, the concordance was moderate–good (kappa index: 0.607); of the 31 cases revealed by FISH, 8 (6.3%) were not seen by MLPA. The percentage of tumor cells with this alteration was low (range: 10-50%). FISH did not detect 2p gain in 11 (8.7%) of the 34 cases detected by MLPA. These 11 cases included 3 samples with heterogeneous *MYCN* amplification detected by FISH.

For detection of 11q deletion and 17q gain, identical

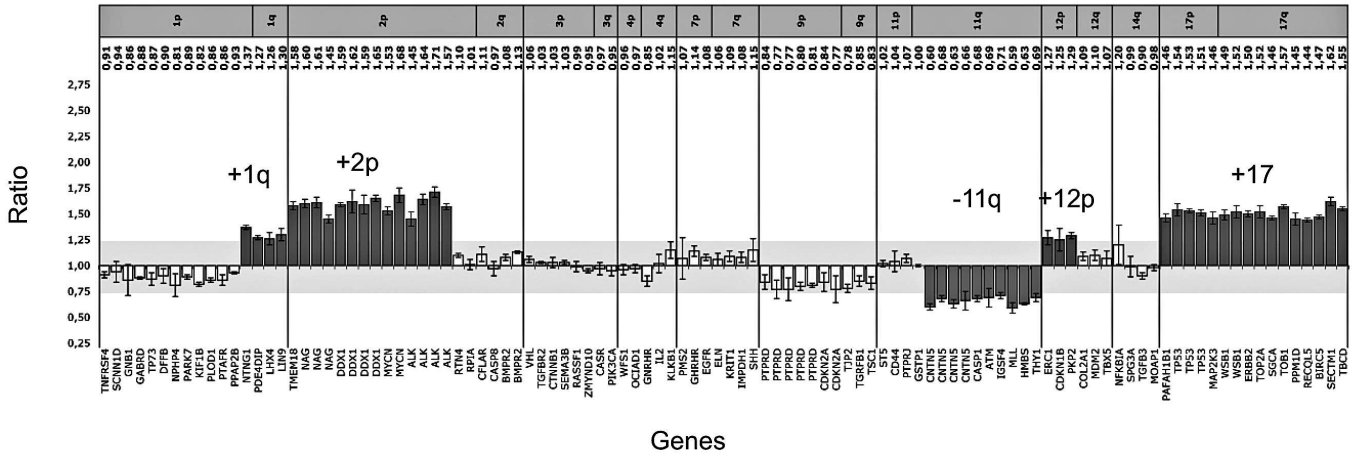

Figure 1. Results using SALSA MLPA Neuroblastoma Kits (A1). The thresholds for loss and gain detection were set at 0.75 and 1.25, respectively. Gene gains indicated in dark up bars were considered chromosome aberrations (+1q; +2p; +12p; +17). Gene losses indicated in dark down bars were considered chromosome aberrations (-11q).

results were obtained by FISH and MLPA in 96.8% and 96% of the cases respectively. The concordance between techniques was very good in both cases (kappa index: 0.908 and 0.922 respectively).

In cases of low concordance, we studied those parameters that may have affected the results. The percentage of neuroblastic cells was determined by HE, analyzing the effect of this parameter on the concordance between results of FISH and MLPA techniques. The mean percentage of neuroblastic cells in the study samples was 68.9±16.7. Table 2 shows the number of cases with genetic alteration detected by each

Table 1. Results obtained by FISH and MLPA, kappa index and concordance between both techniques in detecting the alterations studied.

Alteration	Technique		MLPA		Kappa Index (p-value)	Concordance (Scale of agreement)
			No	Yes		
1p deletion	FISH	No	80 (62.5%)	3 (2.3%)	0.610 (>0.001)	Good (0.61-0.80)
		Yes	18 (14.1%)	27 (21.1%)		
1p imbalance	FISH	No	109 (85.8%)	8 (6.3%)	0.132 (0.138)	Poor (<0.20)
		Yes	8 (6.3 %)	2 (1.6%)		
MYCN amplification	FISH	No	112 (85.7%)	0 (0%)	0.745 (<0.001)	Good (0.61-0.80)
		Yes	6 (4.7%)	10 (7.8%)		
2p gain	FISH	No	85 (66.9%)	11 (8.7%)	0.607 (<0.001)	Moderate/Good (0.41-0.60/0.61-0.80)
		Yes	8 (6.3%)	23 (18.1%)		
11q deletion	FISH	No	90 (70.9%)	2 (1.6%)	0.908 (<0.001)	Very good (0.81-1.00)
		Yes	2 (1.6%)	33 (25.9%)		
17q gain	FISH	No	72 (56.6%)	0 (0%)	0.922 (<0.001)	Very good (0.81-1.00)
		Yes	5 (4%)	50 (39.4)		

Table 2. Effect of percentage of tumor cells on concordance between FISH and MLPA.

Alteration			% Neuroblastic cells					
			40-50% (n=26)		51-60% (n=18)		>60% (n=85)	
			MLPA		MLPA		MLPA	
			No	Yes	No	Yes	No	Yes
1p deletion	FISH	No	15 (57.7%)	2 (7.7%)	4 (22.2%)	5 (27.8%)	56 (66.7%)	1 (1.2%)
		Yes	3 (11.5%)	6 (23.1%)	9 (50%)	0 (0%)	11 (13.1%)	16 (19%)
	Kappa index (p-value)		0.564 (0.004)		0.556 (0.009)		0.637 (<0.001)	
MYCN amplification	FISH	No	24 (92.3%)	0 (0%)	14 (77.8%)	0 (0%)	74 (88.1%)	0 (0%)
		Yes	1 (3.8%)	1 (3.8%)	2 (11.1%)	2 (11.1%)	3 (3.6%)	7 (8.3%)
	Kappa index (p-value)		0.649 (<0.001)		0.609 (0.005)		0.804 (<0.001)	
2p gain	FISH	No	18 (69.2%)	2 (7.7%)	11 (61.1%)	2 (11.1%)	56 (67.5%)	7 (8.4%)
		Yes	0 (0%)	6 (23.1%)	2 (11.1%)	3 (16.7%)	6 (7.2%)	14 (16.9%)
	Kappa index (p-value)		0.806 (<0.001)		0.446 (0.058)		0.579 (<0.001)	

Table 3. Effect of numeric alterations on concordance between FISH and MLPA.

Alteration			Absence of numeric alterations		Presence of numeric alterations	
			MLPA		MLPA	
			No	Yes	No	Yes
1p deletion	FISH	No	10 (25%)	0 (0%)	70 (79.5%)	3 (3.4%)
		Yes	8 (20%)	22 (55%)	10 (11.4%)	5 (5.7%)
	Kappa index (p-value)		0.579 (<0.001)		0.359 (<0.001)	
MYCN amplification	FISH	No	18 (62.1%)	0 (0%)	94 (95%)	0 (0%)
		Yes	2 (6.9%)	9 (31%)	4 (4%)	1 (1%)
	Kappa index (p-value)		0.848 (<0.001)		0.322 (<0.001)	
2p gain	FISH	No	15 (51.7%)	1 (3.4%)	70 (71.4%)	10 (10.2%)
		Yes	2 (6.9%)	11 (37.9%)	6 (6.1%)	12 (12.2%)
	Kappa index (p-value)		0.789 (<0.001)		0.499 (<0.001)	

Table 4. Effect of disomic cells on concordance between FISH and MLPA.

Alteration			Absence cells 2:2		Presence cells 2:2	
			MLPA		MLPA	
			No	Yes	No	Yes
1p deletion	FISH	No	4 (30.8%)	0 (0%)	76 (66.1%)	3 (2.6%)
		Yes	2 (15.4%)	7 (53.8%)	16 (13.9%)	20 (17.4%)
	Kappa index (p-value)		0.683 (0.009)		0.574 (<0.001)	
MYCN amplification	FISH	No	13 (68.4%)	0 (0%)	99 (90.8%)	0 (0%)
		Yes	1 (5.3%)	5 (26.3%)	5 (4.6%)	5 (4.6%)
	Kappa index (p-value)		0.872 (<0.001)		0.645 (<0.001)	
2p gain	FISH	No	12 (63.2%)	0 (0%)	73 (67.6%)	11 (10.2%)
		Yes	0 (0%)	7 (36.8%)	8 (7.4%)	16 (14.8%)
	Kappa index (p-value)		1 (<0.001)		0.513 (<0.001)	

technique grouped for percentage of neuroblastic cells and kappa index. The kappa index showed that concordance between the techniques in detecting 1p deletion and *MYCN* amplification improves as the percentage of tumor cells increases. For 2p gain, the best concordance was shown in samples with 40-50% of tumor cells.

Whole extracopies of chromosomes 1, 2, 11 and 17 were determined by FISH, and their effect on the detection of segmental aberrations with both techniques also studied. In all cases the concordance was higher when no numeric alterations were present in the tumor cells (Table 3). The effect of chromosomes 1, 2, 11 and 17 disomies in the sample diagnosed by FISH was analyzed (Table 4). The presence of chromosomes 1 and 2 disomies reduced the concordance between FISH and MLPA.

Regarding 11q deletion and 17 gain detection, the concordance between techniques was high and also independent of tumor cell percentage, presence of other numeric alterations or 2:2 cells on the chromosomes studied.

Discussion

An increasing number of molecular markers of value in predicting better survival or response to therapy have been identified for different tumors, including NB. These advances will permit a better choice of therapy. However, accurate risk estimation of the patients is mandatory, and the incorporation of new techniques in routine diagnosis for the simultaneous detection of multiple markers or loci is necessary. MLPA appears very promising for routine diagnosis and has been included in the diagnosis of various disorders (Ahn et al., 2007; Kriek et al., 2007). Specifically in NB, with the appropriate kit, MLPA allows the simultaneous detection of DNA copy number of 115 loci. However, before MLPA can be established routinely, it requires validation with other currently-used techniques (Ambros et al., 2009). In a previous study, our group compared the results obtained by MLPA in 20 NB patients with several other techniques used in determining NB DNA copy number alterations, finding a high concordance between them (Villamon et al., 2008). In the present study MLPA was used to detect the genetic alterations in 129 NB patients, comparing the results for five common alterations in NB (1p alteration, *MYCN* amplification, 2p gain, 11q deletion and 17q gain) with those obtained by FISH.

The kappa index was used to determine the concordance between FISH and MLPA, achieving very good results for *MYCN* amplification, 11q deletion and 17q gain. Homogeneous amplification is characterized by *MYCN* amplification in all cells, although a proportion of tumor cells may also show *MYCN* gain (Ambros et al., 2009). This characteristic, combined with a high grade of gene amplification, permitted all cases of homogeneous *MYCN* amplification shown by FISH to be detected by MLPA with perfect concordance. Previously, Ambros et al., (2003) showed that heterogeneous *MYCN* amplification was not detected by DNA-extraction methods. MLPA detection of heterogeneous amplification is possible, but depends on the percentage of amplified tumor cells in the sample and their grade of amplification. In this study the samples with *MYCN* amplification detected by FISH but not by MLPA were cases with heterogeneous *MYCN* amplification. These cases presented only 5-10% of tumor cells with this alteration, and were either not detected by MLPA or were detected as 2p gain.

In our experience, FISH results have shown that 11q deletion and 17q gain are present in a high percentage of tumor cells. In this study, all the samples analyzed had a high percentage of neuroblasts with these segmental aberrations and were therefore easily detected by MLPA.

Previous studies have shown good concordance between FISH and molecular biology techniques in detecting *MYCN* status and 1p36 deletion (Ambros et al., 2001). In the present study there was a good concordance in detecting 1p deletion, although some cases had 1p deletion shown by FISH that were not detected by MLPA because the alteration was present in only 10% to 30% of the tumor cells. These minimal

percentages of neuroblastic cells with 1p deletion were insufficient for MLPA detection of this alteration. MLPA detected one case as 1p deletion that was considered 1p imbalance by FISH. Such discrepancies may be caused by tumor heterogeneity related to the presence or absence of these aberrations. Furthermore, it is possible that this case presented a microdeletion in 1p36 that could not be detected by FISH. It is not possible to detect microaberrations by classical cytogenetic techniques such as FISH that have a resolution of 5-500 kb. To detect these aberrations, techniques with higher resolution such as aCGH, SNPs or MLPA are necessary (George et al., 2005; Kriek et al., 2007; Savola et al., 2007; Ambros et al., 2009; Noguera et al., 2010).

Regarding detection of 1p imbalance, there was low concordance between the techniques. The only two concordant cases presented a high percentage of tumor cells with the 1p imbalance (70-80%). In the other 6 cases detected by FISH but not by MLPA 10% to 60% of tumor cells presented this imbalance. A high number of cases with 1p imbalance detected by MLPA corresponded to cases with high intratumor heterogeneity detected by FISH, with some clones with 1p deletion, some with whole extracopies of chromosome 1 plus clones showing 1p imbalance.

The gain of the *MYCN* gene detected by MLPA (exon 3: probes 2572-L9025 = 372 pb and probes 3327-L2466 = 436 pb) and the absence of extra *MYCN* signals of DNA-FISH probes (D2S2676-RH112907 = 380 Kb), indicate that a cryptic copy number of some exons of the *MYCN* gene was present. This type of aberration can be detected using MLPA, which has a higher resolution than FISH and significantly reduces the time and cost of analysis. The heterogeneity of the samples, arising from a variation in the quantity of tumor and Schwann cells, and from a variation in tumor cells with 2p gain, or *MYCN* gene amplification, and/or extra copy number of some exons of *MYCN* gene would explain the discrepancies between the techniques in detecting *MYCN* gene aberrations.

MLPA is based on PCR and is sensitive to contamination by DNA of normal cells. To use PCR and Southern blot techniques in NB, a sample tumor cell percentage of about 60% is recommended to obtain good results (Ambros and Ambros, 2001; Ambros et al., 2003). In breast cancer *HER2* amplification has been detected by MLPA, finding the best correlation with FISH when the tumor cell percentage was higher than 30% (Moelans et al., 2009). Our experience has shown that at least 40% neuroblastic cells is required to obtain concordant results between FISH and MLPA when all the neuroblasts present the segmental chromosome alteration and if these are not combined with a high number of neuroblasts with numerical aberrations in the affected chromosome. In all cases, except those with 2p gain, the concordance between techniques was higher when the tumor cell percentage in the sample was >60%. The lowest kappa index value was obtained when the tumor cell percentage was between 51% and 60%, this being due to the limited number of samples in this group. In the case of 2p gain, the highest kappa index was found when the percentage of tumor cells was 40-50% because this group contained a low number of samples, without intratumoral heterogeneity and with low percentage of numeric alteration of chromosome 2.

NB has a high genetic and intratumoral heterogeneity, frequently finding various clones of cells with different numeric chromosome variations when a specific gene and the reference are studied by FISH (Ambros et al., 2001; Theissen et al., 2009). MLPA presents difficulties in the interpretation of the numeric alterations status of each chromosome if the reference or control chromosomes of the respective probe set are not disomic. In our study 65-80% of the samples presented numeric chromosome aberrations in addition to segmental ones, especially for chromosomes 1 and 2. Absence of numeric alterations detected by FISH resulted in a good concordance between techniques.

The kappa index was good when disomic cells (2:2 signal for all chromosomes analyzed) were present, but improved when stromal or disomic tumor cells were absent. Disomic cells were present in 80-90% of the samples, but the percentage of 2:2 cells was low in the majority of these because they presented >60% of tumor cells and these tumor cells are rarely 2:2.

The discrepancies between techniques found in this study, including high concordance for one chromosome but not another, could be the result of intratumoral heterogeneity observed when different tumor areas are analyzed by FISH and/or MLPA, as well as to the low percentage of neuroblastic cells with the specific DNA copy number alteration, the presence of a high number of neuroblasts with numeric aberrations, a high level of disomic cells and small extracopy or microdeletion chromosome rearrangements for all chromosome analyzed. A minimum percentage of neuroblastic cells with a specific aberration is necessary to reliably detect segmental aberration by MLPA. For this reason a tumor cell content of 60% is recommended to obtain good results by MLPA.

In conclusion, MLPA, using specific kits with probes designed for genes located in the 10 chromosome regions of greater interest in NB, is an alternative multigenetic technique. It is quite robust, easy to perform, and can rapidly screen a large number of samples. The FISH technique is important for visualizing isolated cells, detecting alterations present in a low percentage of tumor cells, and is essential for the mandatory study of *MYCN* status. The incorporation of MLPA in routine diagnosis will be interesting in detecting the implication of the common genetic alterations in NB. It will provide important information for the estimation of prognosis and selection of therapy for patients, and in the delineation of critical regions of gain and loss in NB, which should facilitate further selection of candidate oncogenes or tumor suppressor

genes.

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References

Ahn J.W., Ogilvie C.M., Welch A., Thomas H., Madula R., Hills A., Donaghue C. and Mann K. (2007). Detection of subtelomere imbalance using MLPA: validation, development of an analysis protocol, and application in a diagnostic centre. BMC Med. Genet. 8, 9.

Alibakhshi R., Kianishirazi R., Cassiman J.J., Zamani M. and Cuppens H. (2008). Analysis of the CFTR gene in Iranian cystic fibrosis patients: identification of eight novel mutations. J. Cyst. Fibros. 7, 102-109.

Ambros P.F. and Ambros I.M. (2001). Pathology and biology guidelines for resectable and unresectable neuroblastic tumors and bone marrow examination guidelines. Med. Pediatr. Oncol. 37, 492-504.

Ambros P.F., Ambros I.M., Kerbl R., Luegmayr A., Rumpler S., Ladenstein R., Amann G., Kovar H., Horcher E., De Bernardi B., Michon J. and Gadner H. (2001). Intratumoural heterogeneity of 1p deletions and *MYCN* amplification in neuroblastomas. Med. Pediatr. Oncol. 36, 1-4.

Ambros I.M., Benard J., Boavida M., Bown N., Caron H., Combaret V., Couturier J., Darnfors C., Delattre O., Freeman-Edward J., Gambini C., Gross N., Hattinger C.M., Luegmayr A., Lunec J., Martinsson T., Mazzocco K., Navarro S., Noguera R., O'Neill S., Potschger U., Rumpler S., Speleman F., Tonini G.P., Valent A., Van Roy N., Amann G., De Bernardi B., Kogner P., Ladenstein R., Michon J., Pearson A.D. and Ambros P.F. (2003). Quality assessment of genetic markers used for therapy stratification. J. Clin. Oncol. 21, 2077-2084.

Ambros P.F., Ambros I.M., Brodeur G.M., Haber M., Khan J., Nakagawara A., Schleiermacher G., Speleman F., Spitz R., London W.B., Cohn S.L., Pearson A.D. and Maris J.M. (2009). International consensus for neuroblastoma molecular diagnostics: report from the International Neuroblastoma Risk Group (INRG) Biology Committee. Br. J. Cancer 100, 1471-1482.

Betts D.R., Cohen N., Leibundgut K.E., Kuhne T., Caflisch U., Greiner J., Traktenbrot L. and Niggli F.K. (2005). Characterization of karyotypic events and evolution in neuroblastoma. Pediatr. Blood Cancer 44, 147-157.

Boensch M., Oberthuer A., Fischer M., Skowron M., Oestreich J., Berthold F. and Spitz R. (2005). Quantitative real-time PCR for quick simultaneous determination of therapy-stratifying markers *MYCN* amplification, deletion 1p and 11q. Diagn. Mol. Pathol. 14, 177-182.

Caren H., Erichsen J., Olsson L., Enerback C., Sjoberg R.M., Abrahamsson J., Kogner P. and Martinsson T. (2008). High-resolution array copy number analyses for detection of deletion, gain, amplification and copy-neutral LOH in primary neuroblastoma tumors: four cases of homozygous deletions of the CDKN2A gene. BMC Genomics 9, 353.

Caron H., van Sluis P., Buschman R., Pereira do Tanque R., Maes P., Beks L., de Kraker J., Voute P.A., Vergnaud G., Westerveld A., Slater R. and Versteeg R. (1996). Allelic loss of the short arm of chromosome 4 in neuroblastoma suggests a novel tumour suppressor gene locus. Hum. Genet. 97, 834-837.

Carr J., Bown N.P., Case M.C., Hall A.G., Lunec J. and Tweddle D.A. (2007). High-resolution analysis of allelic imbalance in neuroblastoma cell lines by single nucleotide polymorphism arrays. Cancer Genet. Cytogenet 172, 127-138.

Castel V., Canete A., Navarro S., Garcia-Miguel P., Melero C., Acha T., Navajas A. and Badal M.D. (2001). Outcome of high-risk neuroblastoma using a dose intensity approach: improvement in initial but not in long-term results. Medical Pediatr. Oncol. 37, 537-542.

Cohn S.L., Pearson A.D., London W.B., Monclair T., Ambros P.F., Brodeur G.M., Faldum A., Hero B., Iehara T., Machin D., Mosseri V., Simon T., Garaventa A., Castel V. and Matthay K.K. (2009). The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. J. Clin. Oncol. 27, 289-297.

Chen Q.R., Bilke S., Wei J.S., Whiteford C.C., Cenacchi N., Krasnoselsky A.L., Greer B.T., Son C.G., Westermann F., Berthold F., Schwab M., Catchpoole D. and Khan J. (2004). cDNA array-CGH profiling identifies genomic alterations specific to stage and *MYCN*-amplification in neuroblastoma. BMC Genomics 5, 70.

Erlandson A., Samuelsson L., Hagberg B., Kyllerman M., Vujic M. and Wahlstrom J. (2003). Multiplex ligation-dependent probe amplification (MLPA) detects large deletions in the MECP2 gene of Swedish Rett syndrome patients. Genet. Test 7, 329-332.

George R.E., London W.B., Cohn S.L., Maris J.M., Kretschmar C., Diller L., Brodeur G.M., Castleberry R.P. and Look A.T. (2005). Hyperdiploidy plus nonamplified *MYCN* confers a favorable prognosis in children 12 to 18 months old with disseminated neuroblastoma: a Pediatric Oncology Group study. J. Clin. Oncol. 23, 6466-6473.

Hogervorst F.B., Nederlof P.M., Gille J.J., McElgunn C.J., Grippeling M., Pruntel R., Regnerus R., van Welsem T., van Spaendonk R., Menko F.H., Kluijt I., Dommering C., Verhoef S., Schouten J.P., van't Veer L.J. and Pals G. (2003). Large genomic deletions and duplications in the BRCA1 gene identified by a novel quantitative method. Cancer Res. 63, 1449-1453.

Janoueix-Lerosey I., Schleiermacher G., Michels E., Mosseri V., Ribeiro A., Lequin D., Vermeulen J., Couturier J., Peuchmaur M., Valent A., Plantaz D., Rubie H., Valteau-Couanet D., Thomas C., Combaret V., Rousseau R., Eggert A., Michon J., Speleman F. and Delattre O. (2009). Overall genomic pattern is a predictor of outcome in neuroblastoma. J. Clin. Oncol. 27, 1026-1033.

Jeuken J., Cornelissen S., Boots-Sprenger S., Gijsen S. and Wesseling P. (2006). Multiplex ligation-dependent probe amplification: a diagnostic tool for simultaneous identification of different genetic markers in glial tumors. J. Mol. Diagn. 8, 433-443.

Kriek M., Knijnenburg J., White S.J., Rosenberg C., den Dunnen J.T., van Ommen G.J., Tanke H.J., Breuning M.H. and Szuhai K. (2007). Diagnosis of genetic abnormalities in developmentally delayed patients: a new strategy combining MLPA and array-CGH. Am. J. Med. Genet. A 143, 610-614.

Landis R. and Koch G. (1977). The measurement of observer agreement for categorical data. Biometrics 33, 671-679.

Moelans C.B., de Weger R.A., van Blokland M.T., Ezendam C., Elshof S., Tilanus M.G. and van Diest P.J. (2009). HER-2/neu amplification testing in breast cancer by multiplex ligation-dependent probe amplification in comparison with immunohistochemistry and *in situ* hybridization. Cell Oncol. 31, 1-10.

Monclair T., Brodeur G.M., Ambros P.F., Brisse H.J., Cecchetto G., Holmes K., Kaneko M., London W.B., Matthay K.K., Nuchtern J.G., von Schweinitz D., Simon T., Cohn S.L. and Pearson A.D. (2009). The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report. J. Clin. Oncol. 27, 298-303.

Nakagawa H., Hampel H. and de la Chapelle A. (2003). Identification and characterization of genomic rearrangements of MSH2 and MLH1 in Lynch syndrome (HNPCC) by novel techniques. Hum. Mutat. 22, 258.

Noguera R., Villamon E., Berbegall A., Machado I., Giner F., Tadeo I., Navarro S. and Llombart-Bosch A. (2010). Gain of *MYCN* region in a Wilms tumor-derived xenotransplanted cell line. Diagn. Mol. Pathol. 19, 33-39.

Sartelet H., Grossi L., Pasquier D., Combaret V., Bouvier R., Ranchere D., Plantaz D., Munzer M., Philip T., Birembaut P., Zahm J.M., Bergeron C., Gaillard D. and Pasquier B. (2002). Detection of N-myc amplification by FISH in immature areas of fixed neuroblastomas: more efficient than Southern blot/PCR. J. Pathol. 198, 83-91.

Savola S., Nardi F., Scotlandi K., Picci P. and Knuutila S. (2007). Microdeletions in 9p21.3 induce false negative results in CDKN2A FISH analysis of Ewing sarcoma. Cytogenet. Genome Res. 119, 21-26.

Scaruffi P., Coco S., Cifuentes F., Albino D., Nair M., Defferrari R., Mazzocco K. and Tonini G.P. (2007). Identification and characterization of DNA imbalances in neuroblastoma by high-resolution oligonucleotide array comparative genomic hybridization. Cancer Genet. Cytogenet. 177, 20-29.

Schleiermacher G., Michon J., Huon I., d'Enghien C.D., Klijanienko J., Brisse H., Ribeiro A., Mosseri V., Rubie H., Munzer C., Thomas C., Valteau-Couanet D., Auvrignon A., Plantaz D., Delattre O. and Couturier J. (2007). Chromosomal CGH identifies patients with a higher risk of relapse in neuroblastoma without *MYCN* amplification. Br. J. Cancer 97, 238-246.

Schouten J.P., McElgunn C.J., Waaijer R., Zwijnenburg D., Diepvens F. and Pals G. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res. 30, e57.

Selzer R.R., Richmond T.A., Pofahl N.J., Green R.D., Eis P.S., Nair P., Brothman A.R. and Stallings R.L. (2005). Analysis of chromosome breakpoints in neuroblastoma at sub-kilobase resolution using fine-tiling oligonucleotide array CGH. Genes Chromosomes Cancer 44, 305-319.

Theissen J., Boensch M., Spitz R., Betts D., Stegmaier S., Christiansen H., Niggli F., Schilling F., Schwab M., Simon T., Westermann F., Berthold F. and Hero B. (2009). Heterogeneity of the *MYCN* oncogene in neuroblastoma. Clin. Cancer Res. 15, 2085-2090.

Vandesompele J., Baudis M., De Preter K., Van Roy N., Ambros P., Bown N., Brinkschmidt C., Christiansen H., Combaret V., Lastowska M., Nicholson J., O'Meara A., Plantaz D., Stallings R., Brichard B., Van den Broecke C., De Bie S., De Paepe A., Laureys G. and Speleman F. (2005). Unequivocal delineation of clinicogenetic subgroups and development of a new model for improved outcome prediction in neuroblastoma. J. Clin. Oncol. 23, 2280-2299.

Villamon E., Piqueras M., Mackintosh C., Alonso J., de Alava E., Navarro S. and Noguera R. (2008). Comparison of different techniques for the detection of genetic risk-identifying chromosomal gains and losses in neuroblastoma. Virchows Arch. 453, 47-55.

Wan T.S., Ma E.S., Chan G.C. and Chan L.C. (2004). Investigation of *MYCN* status in neuroblastoma by fluorescence *in situ* hybridization. Int. J. Mol. Med. 14, 981-987.

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