Assessment of MYCN amplification status in Tunisian Neuroblastoma: CISH and MLPA combining approach.

Évaluation de l'amplification MYCN dans les Neuroblatomes Tunisiens: approche comparative CISH et MLPA.

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RÉSUMÉ

Prérequis : Le Neuroblastome est un modèle oncogénique qui concrétise l'apport des études fondamentales notamment génétiques aux applications cliniques. Ces tumeurs montrent plusieurs altérations génétiques généralement de mauvais pronostic et nécessite par conséquent une stratégie thérapeutique plus intensive. L'amplification de MYCN est le facteur génétique majeur de mauvais pronostic qui témoigne d'une agressivité tumorale.

But : 1) Apporter une stratégie comparative et efficace moyennant deux techniques moléculaires CISH et MLPA afin de révéler le statut du gène MYCN.
2)Etablir une corrélation entre l'évolution clinique et les résultats génétique chez 15 patients porteurs de tumeurs neuroblastiques.

Méthodes : Au cours de ce travail nous avons entrepris une étude transversale chez 15 patients ayant consulté durant la période allant de 2004 à 2011 et pour lesquels nous avons pu récupérer les échantillons tumoraux frais et FFPE.

Résultat : Bien que la survie globale des patients étudiés ne corrèlent pas statistiquement avec leur classifications clinique (COG), une forte concordance entre les deux techniques CISH et MLPA dans la révélation de l'amplification de MYCN a été confirmé (coefficient Kappa= 0.02).

Conclusion : Ce travail nous a par ailleurs permis de conclure quant à la pertinence de la stratégie moléculaire adoptée. Il s'agit en effet et conformément aux recommandations internationales d'une stratégie qui combine deux techniques moléculaires : CISH et MLPA. L'utilisation simultanée de ces 2 techniques constitue une stratégie de routine adoptée au sein de notre laboratoire avec but ultime d'adapter la stratégie thérapeutique des tumeurs neuroblastiques Tunisiennes.

Mots-clés
Neuroblastome, MYCN, CISH, Pronostique, Génétique

SUMMARY

Background: Neuroblastoma (NB) shows a complex combination of genetic aberrations. Some of them represent poor genetic prognosis factors that require specific and intensive chemotherapy. MYCN amplification consists of the major bad outcome prognostic factor, it is indeed frequently observed in aggressive neuroblastomas. To date different methods are used for MYCN status detection.

Objectives: The primary aim of our study was to provide a critical assessment of MYCN status using 2 molecular techniques CISH and MLPA. We also focused on the correlation between neuroblastoma genetic markers and patient's clinical course among 15 Tunisian patients.

Methods: we developed a descriptive study that includes 15 pediatric Tunisian patients referred to our laboratory from 2004 to 2011. We reported the analysis of fresh and FFPE NB tumors tissues.

Results: No significant correlation was found between COG grade and patients overall survival. Assessment of NMYC gene copy number by kappa statistic test revealed high concordance between CISH and MLPA tests (kappa coefficient = 0.02).

Conclusion: Despite misdiagnosing of MYCN status fewer than 5 copies, MLPA remains an effective molecular technique that enables a large panel of genomic aberrations screening. Thus combining CISH and MLPA is an effective molecular approach adopted in our laboratory. Our results allow pediatric oncologists to set up the first Neuroblastoma therapeutic strategy based on molecular markers in Tunisia.

Key-words
Neuroblastoma, MYCN, CISH, Prognosis, Genetics

Neuroblastoma (NB) is a malignant embryonal extra-cranial tumor that develops in the sympathetic nervous system (1). It commonly occurs in pediatric patients from birth to 10 years old. Currently COG classification (children's oncology group classification) consists of the major established prognosis staging systems of neuroblastoma. Among its criteria, COG considers the INSS (International Neuroblasto ma Staging System) postsurgical staging system (2). Recent studies showed that, Neuroblastoma prognosis could be predicted through the tumor molecular profile. Different genetic aberrations have been described and associated with poor prognosis : MYCN amplification (3), 1p loss (4), 11q loss(5) and 17q gain(6). Thus it requires more intensive chemotherapy (7). For this reason, Neuroblastoma genetic profile should be characterized in timely fashion that can help for therapeutic decision. Today, different laboratory methods are used for screening of genetic abnormalities. Those techniques must be precise quick and normalized, because of the results importance. The primary aim of our study was to compare two molecular techniques used in screening the genetic modifications in Tunisian Neuroblastoma tumors. There by, we adopt a chromogenic in situ hybridization (CISH) and multiplex ligation probe amplification (MLPA). We investigated their efficiency and limits in Neuroblastoma prognosis definition. Finally, we assessed the patients survival based on the therapeutic strategy adopted after molecular analysis.

METHODS

Patients:

We studied 15 tumors from genetically unrelated Tunisian patients. Their age at diagnosis ranged from 2 days to 10 years. Based on COG classification, 7 patients were classified as poor prognosis (age group>18 months). The 8 others (<18 months) had a better prognosis (2, 8) (Table 1).

Tissues collection

A total of 15 archival FFPE (formalin fixed paraffin embedded) Neuroblastomas from year 2004 to 2011 had been collected in the department of Histopathology of Monastir, Tunisia. Histological diagnosis and grading were established and reviewed is the same department based on the international Neuroblastoma pathology classification (INPC). The tumor collection was composed by 12 cases of Neuroblastoma (NB), 2 Ganglioneuroblastoma (GNB) and one Ganglioglioma (GN). All analyzed tissues except two cases of reccurence (NB #10 and 12) derived from primary tumors obtained at diagnosis without previous chemotherapy. The overall free survival (OFS), the overall survival (OS) and the follow up were conducted by reviewing patient's medical records. The clinical stage was evaluated according to the INSS classification (Table 1).

CISH technique

2 µm from each block were cut and spread over a superfrost slides for CISH analysis. CISH is a molecular quantitative technique based on the capacity of denatured DNA probes to hybridize with complementary genomic denatured DNA. MYCN amplification was investigated with a commercial probe (ZytoDot SPE myc-N Probe Kit) according to manufacturer recommended protocol. The adopted technique did not allow combining MYCN probe with a reference probe on chromosome 2. As maintained by ZytoDot SPE myc-N Probe Kit, the average of signals in 30 cells per section, at least, was calculated. Tumors with high amplification show a large cluster of the gene in more than 50% of the cancer cells (Fig. 1c, fig.1d)(9) . Sections with 6 to 10 copies per nucleus are also considered as amplified MYCN tumors (10) but the presence of 3 to 5 copies of MYCN may be due to polysomy and they should be interpreted as negative for MYCN amplification (Fig. 1b) .

Table 1 : CISH and MLPA analysis of MYCN, 2p, lp and 17 q copy number status

Table 1: CISH and MLPA analysis of MYCN,2p, 1p and 17q copy number status

NB#	INSS	Age at diagnosis	CISH MYCN amplification status	MLPA copy number modifications					Histology	OFS	OS	Patient follow-up	COG risk group
				1p	2p	7q	11q	17q					
5	4	7 M	MYCN Clusters	N	gain	N	N	gain	NB	1 month	1 month	metastasis after a few months	high
6	4	20 M	MYCN Clusters	loss	gain	N	N	gain	GNB	months	months	dead	high
8	1	10 M	MYCN Clusters	N	gain	N	loss	gain	GNB	8years	8years	no recurrence no metastasis	low
1	4	28 D	9 MYCN copies	N	gain	N	N	gain	NB	months	months	metastasis at the time of diagnosis	high
10	4	10 Y	8 MYCN copies	N	gain	N	N	gain	NB	6years	6years	recurrence	high
9	3	5 Y	6 MYCN copies	N	gain	N	N	gain	NB	21months	27months	dead	high
7	2B	3 Y	6 MYCN copies (heterogen)	loss	N	N	loss	N	NB	12 months	30 months	dead	low
4	3	8 M	5 MYCN copies	N	N	N	loss	gain	NB	6years	6years	no recurrence no metastasis	int
8	3	4 Y	5 MYCN copies	loss	N	gain	N	N	NB	4years	4years	no recurrence no metastasis	high
2	3	6 M	5 MYCN copies	ND					GN	3years	3years	no recurrence no metastasis	int
13	4s	1 M	ND (Benin)	loss	gain	N	loss	gain	NB	months	months	dead	high
11	4	4 Y	Normal status	loss	N	N	N	gain	NB	14 months	14 months	metastasis at the time of diagnosis dead	high
12	3	4 Y	Normal status	loss	N	N	N	gain	NB	4 months	5 months	recurrence and metastasis	high
14	4	5 M	Normal status	N	N	N	loss	N	NB	1year	1 year	metastasis at the time of diagnosis dead	int
15	4	31 M	Normal status	ND					NB	1year	1year	metastasis after a few months Dead	int

Footnotes: ND: not determined / gain: amplification/ loss: deletion / N: normal status /m : months/D: days/ y: years/ NB: neuroblastomas/ GN: ganglioneuromas/ GNB: ganglioneuroblastomas/ int: intermediate.

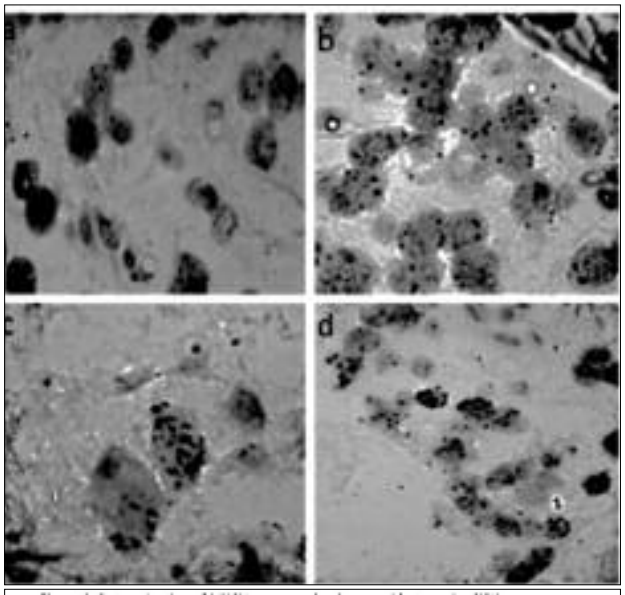

Figure 1: Determination of MYCN copy number in neuroblastoma by CISH with DAB and hematoxylin counterstain
(1.a) a tumor that is diploid for MYCN with 2 signals per nucleus (X60)
(1.b) a tumor with low level amplification for MYCN (magnification X60).
(1.c) a tumor with high level amplification for MYCN (magnification X60).
(1.d) a tumor with high level amplification for MYCN (magnification X 100).

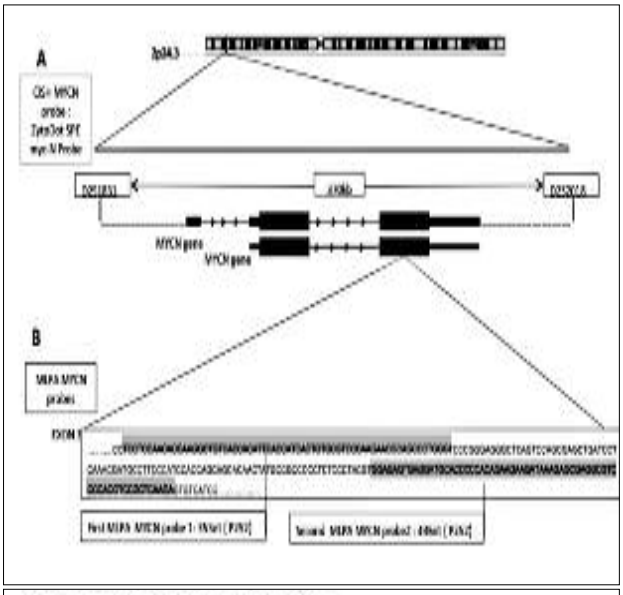

Figure 2 : CISH and MLPA MYCN probes locations
(A): MYCN CISH probe location (gray).
(B): MYCN MLPA probe location , MYCN MLPA probes are indicated by dark and light gray rectangles. P252 MLPA kit presents probe 1 and 2 sizes with 453 and 486 nucleotides respectively.

MLPA

For each FFPE tumor, DNA was extracted from 8 sections at 5 µm, using QIAamp DNA FFPE Tissue Kit (Qiagen). DNA extraction was performed according to manufacturer protocol. When fresh sample was available, 50 mg of tissue were digested by proteinase K (10mg/ml) followed by phenol-chloroform extraction according to standard protocols.

MLPA was performed using the SALSA MLPA Kits P251/P252/P253 (version B1), developed by MRC-Holland. MLPA allows estimation in a single reaction of relative copy number of up to 45 nucleic acid sequences in each kit. In each set of MLPA experiments, 10 control probes located in chromosomal regions rarely altered in NB are included. MLPA was performed as described by the manufacturer with minor modifications. Data analysis was performed with "GeneMarker®" software (Soft Genetics) as described by Jeuken et al.; losses and gains thresholds were set at 0.8 and 1.2, respectively . For DNA quality, 5 control probes were included .

Both MLPA and CISH MYCN probes locations details are described in figure 2. ZytoDot SPE CISH Probe covers MYCN gene (Fig.2A). Meanwhile, MLPA present 2 probes located within the gene sequence and specific for the MYCN exon 3 region (Fig.2B). Indeed, MYCN CISH probe is located between microsatellite markers D2S1831 and D2S2018 covering 370 kb and encompassing MYCN MLPA targets.

RESULTS

Based on clinical markers, COG grade and patients follow up led to separate the studied cohort into 2 subgroups: the first subgroup is composed by 6 patients with low grade encompassing low and intermediate COG risk groups. The second subgroup includes 9 patients with high risk COG group. Note that the 6 patients belonging to the first low grade COG were distributed in 4 with OS > 2 years and 2 with OS < 2 years. While high risk COG group was divided into 3 with OS > 2 years and 6 with OS < 2 years. Overall, high risk studied group have less OS than the low risk ones (fig.3A). But, in another point of view, 2 patients with low risk COG have less OS than those classified as high risk. Based on this result it's clear that clinical data cannot absolutely define the prognosis regardless the genetic tumor profile (Fig. 3A).

With regard to both MYCN status and patients OS, 6 of the 8 tumors with MYCN amplification had a bad outcome (OS<2 years). Unexpectedly, 2 of the 8 had more than 2 years OS. Similarly, 3 patients without MYCN amplification, had a bad outcome (Fig.3B). Statistic analysis showed that the group of patients with MYCN amplification had an OS less than those without amplification. But no significant p (value) was found. Those results confirmed that tumor evolution maybe influenced by MYCN statues and clinical factors as well (Age at diagnosis, INSS grade and INPC grade). MYCN amplification remains a good prognosis marker, but cannot be considered alone to predict patient's outcome.

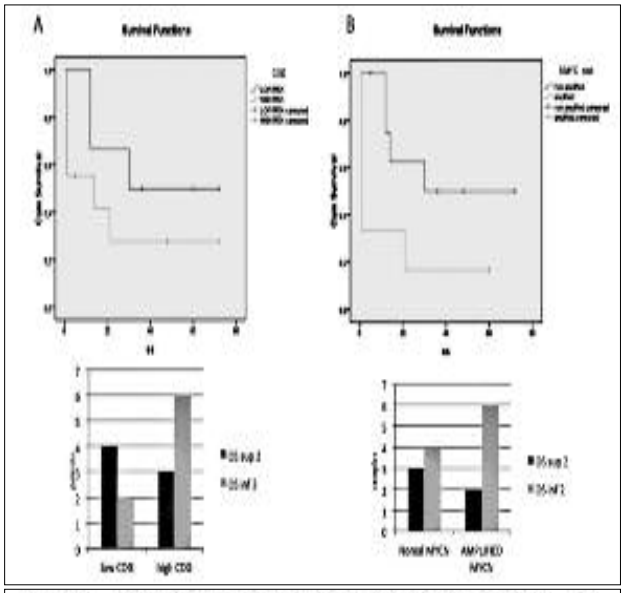

Figure 3 : Impact of MYCN status and COG grade on the Overall survival of a neuroblastoma patients according to kaplan Meier test .SPSS 0.17 (A) Kaplan-Meier estimation of Overall survival for the 15 neuroblastoma samples stratified according to COG classification . (B) Kaplan-Meier estimation of Overal survival for the 15 neuroblastoma samples classified according to MYCN status.

CISH:

CISH was successfully interpreted in 14 of 15 tumors. Only 1 Case (NB# 13), which is an old biopsy (since 2004) fixed in Holland's Bouin, didn't reveal any signal. CISH staining result was interpreted with light microscope using 40X dry lens. It was stained with hematoxylin to allow the evaluation of MYCN status in all individualized tumor cell and scored as described above. Among 14 analyzed primary tumors, we identified a total of 7 (50 %) non amplified, 2 (14.2%) low amplified and 5 (35.7%) tumors with a high MYCN amplification. Detailed MYCN amplification statuses are reported on table 1.

MLPA

MLPA results are summarized in table 1. MLPA was successful in 13 of the 15 tumors (86.66%). Unsuccessful cases corresponded to 2 FFPE old tumor blocks (NB# 2; 15) derived from two patients already treated with neoadjuvant chemotherapy. The highest incidence of genetic imbalance concerned 17q gain in 10 of 13 MLPA analyzed tumors (76%), 7 of them showed also 2p gain. 1p and 11q losses were fond were set at same frequencies (38%) and are dramatically less observed than gain unbalances.

Interestingly using kappa statistic test, assessment of NMYC gene copy number revealed high concordance between CISH and MLPA tests (Kappa coefficient = 0.02).

DISCUSSION

Regarding the statistic analysis, our results confirmed that patient's prognosis and tumor evolution depend equally on clinical data and genetic tumor profile. Molecular investigations of genetic prognosis markers are essential. The MYCN status is not the only important marker but also the other genetic modifications as well. Thereby, the choices of molecular techniques, that detect those genetic aberrations, have to live up to their task, be quick and precise to allow the prognostic prediction with better therapeutic adjustment.

The primary aim of our study was to look for appropriate techniques that could be relevant in our laboratories. We combined, so CISH and MLPA to screen MYCN amplification status in NB. We approved CISH as a simple technique, easy to interpret and relevant for routinely processed specimens. It allows the evaluation of MYCN status in every tumor cell. Nevertheless CISH is a single-color assay that shows only the MYCN probe staining. The use of a second control probe in the same tissue section could be better especially in polyploidy cases. Protocol with dual-color CISH was described recently(11,12). This could be an excellent improvement of CISH flexibility and accuracy.

Although MYCN amplification status is the first tumor genetic marker used in NB therapeutic strategy, it remains insufficient for tumor outcome prediction and prognosis evaluation. For this reason we combined second multigenomic technique to the CISH wish is MLPA. Indeed, MLPA is a multiplexed PCR that screens in the same reaction a large panel of genomic aberrations. It provides the MYCN status with others genetic alterations related to the tumor diagnosis as well as prognosis description. Therefore it allows the better adjustment of the therapeutic strategy.

Meanwhile, MLPA seems to have some disadvantages. Compared to CISH results, MLPA only detected 2p gain in samples with more than 5 signals in CISH slides (NB# 2, 4 and 8).

Knowing that MLPA analyzes pooled cell's DNA, this may explain the misdiagnosis of copy number heterogeneity in the same tumor (NB# 7). MYCN status underestimation may results from diluting out MYCN amplified cells by the non amplified ones in the same tumor. In that case, pediatric oncologists need to be aware of the possibility that poor MYCN amplification and heterogeneous MYCN status may be missed using techniques based on pooled DNA such as CGH or MLPA .

In this regards CISH is an accurate technique. It allows to detect MYCN status in every individualized tumor cell even in cases of tissue heterogeneity. It opens the possibility to correlate MYCN copy number with other cytological parameters. It is interesting to note that both MYCN MLPA targets are designed to cover a specific point located on exon 3 of the gene wish is encompassed by MYCN CISH targets at the mean time (Fig. 2). This led us to look for identical results of MYCN status reveled by CISH and MLPA.

MLPA remains a multigenomic technique. Despite of the misdiagnosing of MYCN status lower than 5 copies, MLPA remains robust, fast, cost effective and easy to perform as previously described (13). Thus the combination of both techniques was adopted as a routine protocol in our laboratory. In the difference to CISH, MLPA technique needs one more day protocol for DNA extraction step. Nevertheless, the most important advantage of MLPA is the possibility of an effective analysis of tumoral DNA extracted from small needle core biopsy and presents the results to oncologist in the shortest delays. That avoids them losing time for therapeutic decisions.

In conclusion, to investigate NB tumors genetic aberrations with a lower cost we adopted: MLPA analysis of needle core or surgical biopsy at diagnosis time followed later by CISH analysis (after tissue fixation) to confirm the MYCN status.

But both techniques reveled specific chromosomal aberrations and not all genetic modifications that NB may present. It's so clear that higher resolution methods, like CGH array, are necessary for a more accurate tumor classification and effective treatment strategies. This study shows that combination of both techniques is an effective molecular approach. Moreover, it should allow pediatric oncologists to set up the first Neuroblastoma therapeutic strategy based on molecular markers in Tunisia.

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