Application of Fluorescence in Situ Hybridization to Detect MYCN Amplification on Paraffin-embedded Tissue Sections of Neuroblastoma - Single Institute Study.

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Abstract

Background

Neuroblastoma is the most common extracranial **solid tumour** in children. Amplification of the *MYCN* gene was observed in approximately 20-30% of tumours. It is strongly correlated with advanced stage of disease, rapid tumour progression, resistant to chemotherapy and poor outcome independent of patient age and stage of advanced disease. Amplification of the *MYCN* identify high-risk patients.

Methods

To assess neuroblastoma tumours with *MYCN* amplification we used paraffin-embedded tissue sections in 57 patients by fluorescent *in situ* hybridization (FISH).

Results

Twenty-eight (49,1%) of patients had localized neuroblastoma (1–3 stages), 5,3% stage 4S, and 45,6% stage 4 disease. Thirty-eight (66,7%) of patients had got abdominal/adrenal mass at diagnosis. Twenty-seven (47,4%) patients were <18 month of age, thirty (52,6%) patients were >18 month of age. *MYCN* amplification appeared in 12 (21%) tumour specimens.

Conclusions

Fluorescent *in situ* hybridization is a high-sensitive, useful technique for detecting *MYCN* amplification on paraffin- embedded tissue section of neuroblastoma tumours thus facilitating therapeutic decisions based on the presence or absence of this important biologic marker.

Background

Neuroblastoma is a malignant tumour in children aged 0-15 years. It accounts for 8-10% of all childhood tumours, 80% concerns children under the age of 5 years and approximately 15% of cancers deaths in children [1, 2]. Approximately 60-70 of the new incidences of each year in Poland are diagnosed [3].

Neuroblastoma derives from primitive cells of the sympathetic nervous system. It occurs in retroperitoneum, posterior mediastinum, the neck or the pelvis. The clinical symptoms in neuroblastoma depend on the tumour mass, the extent of metastases and catecholamine and vasoactive intestinal peptide secretion from tumour cells. The diagnosis of neuroblastoma is based on histopathology test results, the presence tumour cells in bone marrow accompanied by elevated levels of urinary catecholamines. In addition, clinical, histopathological and laboratory data, there are important the genetic test results, in the determining prognosis [4, 5].

The most common cytogenetics changes including deletions of chromosome 1p, amplification of the oncogene *MYCN*, ploidy changes, gains of chromosome arm 17q and deletion of 11q in tumour cells. The

genetic abnormalities are very powerful predictors of response to therapy and outcome and provides important information that guides optimal patient management [6, 7]

The *MYCN* gene is a cellular protooncogene of the *MYC* family. *MYCN* maps to 2p24 chromosome where it is retained in a single copy during amplification. Amplicons containing the *MYCN* gene range in size from 100 kbp to 1 Mbp and form multiple tandem arrays present in two types of aberrant chromatin structures: double minute chromosomes (dmns) and homogeneously staining regions (HSRs). Dmins are often seen as small extra chromosomes in direct metaphase preparations from neuroblastomas, whereas HSRs are integrated as extra segments in abnormal chromosomes and are typically found in established neuroblastoma cell lines [8, 9].

MYCN is an oncogenic transcription factor which function in the regulation of proliferation, transformation, differentiation and apoptosis [1].

Amplification *MYCN* is detected in 20-30% neuroblastoma patients [10, 11]. It is strongly correlated with advanced stage of disease, rapid tumour progression, resistant to chemotherapy and poor outcome independent of patient age and stage of advanced disease. *MYCN* amplification test result is included in the medical protocols throughout the world and it is an independent indicator in the assessment of patient outcome and it is one of the qualifying factors to a group requiring the most aggressive treatment [12, 13, 14, 15, 16]. Amplification *MYCN* is present in 38% of patients with stages 3 and 4 [13] but only 5-10% of patients with stages 1, 2 and 4s [17]. The level of *MYCN* gene amplification is necessary to avoid either under- or over-treating patients [18].

The aim of this study was a standardization of the method to evaluation *MYCN* amplification on paraffin-embedded tissue sections of neuroblastoma patients.

Patients

The test material were paraffin- embedded tissue sections with fresh remove tumours, as well as archival sections 57 patients with an established clinical diagnosis of neuroblastoma (28 females and 29 males; median age: 3 years 2 month). All of the children were admitted to the Department of Pediatric Hematology, Oncology and Transplantology, Children's University Hospital, Lublin, Poland from 1994 to 2019.

The collected clinical data included the patient`s age at diagnosis, stage according to INSS (*International Neuroblastoma Staging System*) criteria [19], tumour location, histopathological diagnosis of the examined tissues and *MYCN* amplification [Table 1]. All studies were performed in the 2002-2019.

Methods

We were used Pretreatment Kit (Vysis) and LSI N-MYC (2p24) SpectrumGreen/CEP 2 SpectrumOrange Probe (Vysis) to estimate *MYCN* amplification.

Paraffin-embedded tissue blocks were cut into 4-micron thick sections on silanized slides and they were baked at 56°C overnight. Slides were soaked twice in xylene for 10 minutes and were dehydrated in ethanol 100%, 96%, 80%, 70% for 2-5 minutes in each solution. Then slides were immersed to 0,2N hydrochloric acid for 20 minutes and were rinsed in water and wash buffer for 3 minutes in each of them. The slides were immersed in Pretreatment Solution at 80°C for 30 minutes and they were washed twice in 2xSSC for 5 minutes.

The next step was digesting tissue sections in protease solution in a 37 °C for 30 minutes and immersed slides in 2xSSC cooled to 5 °C.

Then we were used the 10 % formalin for 10 minutes for fixed tissue, were washed twice in 2xSSC and were dried slides on a 45-50 °C hot plate.

For the detection of the presence of *MYCN* gene amplification, on paraffin- embedded tissue section from neuroblastoma tumour, we have used the fluorescence *in situ* hybridization (FISH) on interphase nuclei. The FISH is the method of molecular cytogenetic, which is used to identify a specific DNA sequences on chromosomes by DNA probes which are direct labelling by fluorophore. The labelled probe and the target DNA are denatured in the first step of this method. The hybridization is the second step of FISH method. Combining the denatured probe and target DNA allows the annealing of complementary DNA sequences.

We were used 1 µl direct fluorochrome- labelled, dual colour DNA Probe for the *MYCN* gene locus (2p24) and α-satellite DNA identifying the centromeric region of chromosome 2 (Vysis LSI N-MYC (2p24) SpectrumGreen/CEP 2 SpectrumOrange Probe) combine with the 7 µl Hybridization Buffer and 2 µl water on the slide. Probe were placed on the slide under a coverslip and ringed rubber cement. The slides were incubated at 72°C for 5 minutes to denature the DNA. Hybridization was done for 14–18 hours in 37°C.

After that the slides were immersed in post- hybridization wash buffer in room temperature for 15-20 minutes until the coverslip dropped out. Then the slides were immersed in post- hybridization wash buffer warmed to 72°C for 5 minutes. Cells were counterstained with DAPI. The signals were scored by eye or captured with Applied Spectral Imaging- ASI- Israel at 100x magnification on the Nikon epifluorescence microscope equipped with a 100-W mercury arc lamp and appropriate set of filters (single filters for FITC and TexasRed fluorochromes and a triple band pass filter for FITC/TR/DAPI). At least 200 nuclei were evaluated for each of the *MYCN* and CEP2 loci. Nuclei with two visibly red and two green signals were scored as a negative *MYCN* amplification (Figure 1). Nuclei with more of 10 green signals, were scored as a positive *MYCN* amplification (Figure 2).

The study was approved by the Ethics Committee of the Medical University of Lublin (KE-0254/229/2012).

Results

Fifty-seven patients participated in this study. Twenty-eight (49,1%) of patients had localized neuroblastoma (1–3 stages), 5,3% stage 4S, and 45,6% stage 4 disease. Thirty-eight (66,7%) of patients had got abdominal/adrenal mass at diagnosis. Twenty-seven (47,4%) patients were <18 month of age, thirty (52,6%) patients were >18 month of age. Of 57 patient samples comprising 43 neuroblastomas, 10 ganglioneuroblastomas, 1 ganglioneuroma. 3 samples had no definitive diagnosis.

Positive results of *MYCN* amplification were observed in twelve patients' paraffin-embedded tissue sections, which represents an 21% of all tested patients in the analysis.

There were 7 (55%) males and 5 (45%) females. Of the 12 patients, 1 (8,33%) was in stage 3 and 1 (8,33%) was in stage 4s disease, 10 (83,33%) were in stage 4 disease. Of these 12 patients with *MYCN* amplifications 4 (33,33%) were <18 month of age, 8 (66,67%) were >18 month of age. Three (25%) of patients with *MYCN* amplification had tumour mass in the abdomen and nine (75%) had tumour location in the adrenal gland.

Discussion

In many trials, high-risk patients are identified by the presence of *MYCN* amplification. Amplification *MYCN* is powerful prognostic factor of advanced stages and rapid tumour progression [20]. Interphase FISH analysis provides a direct and rapid method. Only a small number of tumour cells are required, compared with primary tumour biopsies, and FISH is more sensitive, counting the *MYCN* copy number in each single cell [21, 22].

Fluorescent *in situ* hybridization (FISH) method is faster to prepare than molecular biology techniques or traditional cytogenetic analysis. There is no need to isolation of DNA from tumour cells, which previously could be separate from health tissue. There is no need to take a section of the tumour immediately after the surgery to the FISH study. This can identify mixed cell populations [23, 24]. FISH can be used on interphase nuclei, in contrast to karyotype analysis, and it can be performed on formalin-fixed nuclei, in cases where fresh or frozen tumour is not available (eg. small biopsies or retrospective studies). However molecular methods such as Southern blotting and quantitative DNA polymerase chain reaction (PCR) analyse DNA pooled from a mixture of cells and therefore provide only an average result for a particular tumour. When a tumour is highly amplified, this presents no problem in diagnosis. However, with low levels of *MYCN* amplification, it can be difficult interpretation of results. First it may be a heterogeneous population of tumour cells in which a small amount of cells is highly amplified. Secondly a highly amplified tumour mixed with normal tissue. Thirdly a low level of amplification that occurs in tumour cells (eg. a triploid population). The first two situations would suggest a poor prognosis, whereas triploid tumours have a favourable prognosis. All of them would be regarded as non-amplified for treatment purposes. [25, 26].

The FISH technique also has disadvantages. This method requires the use of specialized equipment to view the slides such as fluorescent microscope with camera and specialized software. Finally, as tumour cells are usually dissociated from each other as part of the FISH technique, information about tissue

architecture is lost, and it is difficult, if not impossible, to distinguish normal cells from tumour cells [26, 27].

In polish population tumour touch imprints are routinely used to assess *MYCN* amplification. However, this is not always possible and then the only material for *MYCN* amplification assessment are paraffin tissue section. The removed tumour is in the whole embedded in paraffin and used for many studies, including histopathological, immunohistochemical and genetic tests. It can be made both on the fresh and archival tissues for many years ago.

For this study we were used fresh paraffin- embedded tissue sections as well as archival materials. A fresh material was made from a tumour which was removed from a patient 2 weeks before the study and archival paraffin section was removed from a patient operated 8 years and 2 months before the test. In our opinion both fresh tissues and those from many years ago preserved in a paraffin block were a full-fledged material for the test. The quality of the preparations was similar and evaluation of both archival and fresh ones was corresponds to previous studies.

In the literatures, high-risk patients had long-term survival of only 10 - 20 % with combination chemotherapy, surgery and local radiation therapy [28]. However, treatment approaches that used a combination of induction therapy, myeloablative consolidation therapy with stem cell support, and biologic therapy had improved 5-year survival rates from less than 15% - 40% [29].

Despite many biochemical, molecular and genetics indicators as a marker of poor prognosis, *MYCN* amplification still feature in patient stratification to the risk group and it is the marker of poor prognosis. These patients respond poorly to conventional therapy. The possibility of determining this biological marker is necessary for appropriate clinical management. Unfortunately, treatment failure has been reported in all patient groups, even without *MYCN* amplification, suggesting additional prognostic markers must be discovered to improve treatments protocols [30].

Declarations

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of the Medical University of Lublin (KE-0254/229/2012).

All of participants have written consent to participate and publish the data. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individuals included in the study and their parents or guardians on behalf of any participant under the age of 16.

Consent for publication

Not applicable.

Availability of data and materials

Data and material are available upon request.

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Competing interests

The authors declare that they have no competing interests.

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Authors' contribution

DW and ML are responsible for the conception and design of the study. JZ shared patients' clinical data. DW and MB conducted laboratory work. BS and PS were responsible for the acquisition of literatures for manuscript. DW and MB were responsible for interpretation of data. DW and ML prepared final manuscript for publication. The final manuscript was reviewed and approved by all authors.

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Tables

Table 1. Characteristics of patients.

Patient	Age at diagnosis	Diagnosis	INSS risk group	Primary site	MYCN amplification
1.	<18 m	NB	3	pelvis	No
2.	>18 m	NB	4	abdomen	No
3.	<18 m	GNB	4	abdomen	No
4.	>18 m	GNB	4	abdomen	No
5.	>18 m	NB	4	abdomen	No
6.	>18 m	NB	4	mediastinum	No
7.	>18 m	GNB	3	adrenal	Yes
8.	<18 m	GNB	1	pelvis	No
9.	<18 m	NB	4	adrenal	Yes
10.	<18 m	NB	3	pelvis	No
11.	>18 m	NB	4	abdomen	Yes
12.	<18 m	GNB	3	mediastinum	No
13.	<18 m	NB	2	mediastinum	No
14.	>18 m	GN	3	mediastinum	No
15.	<18 m	NB	4	pelvis	No
16.	>18 m	NB	2	mediastinum	No
17.	>18 m	NB	3	abdomen	No
18.	>18 m	NB	4	abdomen	No
19.	<18 m	NB	3	adrenal	No
20.	>18 m	NB	4	abdomen	No
21.	<18 m	NB	3	mediastinum	No
22.	<18 m	NB	1	adrenal	No
23.	>18 m	NB	4	abdomen	No
24.	<18 m	NB	3	mediastinum	No
25.	<18 m	NB	4s	cervical	No
26.	<18 m	NB	4	adrenal	Yes
27.	>18 m	GNB	3	mediastinum	No
28.	<18 m	ND	3	mediastinum	No

29.	>18 m	NB	4	abdomen	No
30.	>18 m	NB	4	adrenal	Yes
31.	>18 m	NB	3	pelvis	No
32.	>18 m	NB	1	mediastinum	No
33.	>18 m	NB	4	adrenal	No
34.	>18 m	NB	3	adrenal	Yes
35.	>18 m	NB	3	abdomen	No
36.	>18 m	ND	4	adrenal	Yes
37.	<18 m	NB	4s	adrenal	Yes
38.	>18 m	NB	4	adrenal	Yes
39.	<18 m	NB	3	adrenal	No
40.	<18 m	ND	1	mediastinum	No
41.	<18 m	NB	1	adrenal	No
42.	<18 m	NB	3	pelvis	No
43.	<18 m	NB	4	adrenal	No
44.	>18 m	NB	4	adrenal	Yes
45.	>18 m	GNB	1	adrenal	No
46.	>18 m	NB	3	abdomen	No
47.	<18 m	NB	3	pelvis	No
48.	>18 m	GNB	4	adrenal	No
49.	<18 m	NB	4s	adrenal	No
50.	<18 m	NB	3	adrenal	No
51.	>18 m	GNB	4	adrenal	No
52.	>18 m	NB	3	abdomen	No
53.	>18 m	NB	4	abdomen	Yes
54.	<18 m	NB	4	adrenal	No
55.	<18 m	NB	4	abdomen	Yes
56.	<18 m	NB	4	adrenal	No
57.	>18 m	GNB	4	abdomen	No

M - male, F - female, m-month, NB - neuroblastoma

GNB - ganglioneuroblastoma, GN - ganglioneuroma, ND – no data,

INSS - *International Neuroblastoma Staging System*

Figures

Figure 1

Representative FISH image on paraffin-embedded tissue section using Vysis LSI N-MYC (2p24) SpectrumGreen/CEP 2 SpectrumOrange Probe from neuroblastoma tumour showing normal interphase nuclei captured with Applied Spectral Imaging- ASI-Israel at 100x magnification on the Nikon epifluorescence microscope.

Figure 2

Representative FISH image on paraffin-embedded tissue section using Vysis LSI N-MYC (2p24) SpectrumGreen/CEP 2 SpectrumOrange Probe from neuroblastoma tumour showing amplification MYCN captured with Applied Spectral Imaging- ASI- Israel at 100x magnification on the Nikon epifluorescence microscope.