Fast Detection of MYCN Copy Number Alterations in Brain Neuronal Tumors by Real-Time PCR

S.G. Malakho,^{1*} A. Korshunov,² A.M. Stroganova,³ and A.B. Poltarau¹

¹Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia

²Department of Neuropathology, Burdenko Neurosurgical Institute, Russian Academy of Medical Sciences, Moscow, Russia

³Blokhin Cancer Research Center, Russian Academy of Medical Sciences, Moscow, Russia

Increased *MYCN* gene copy number is a characteristic property of neurogenic tumors. Fluorescence in situ hybridization (FISH) and array-based comparative genomic hybridization (array-CGH) are traditionally used to determine *MYCN* amplification for tumor stratification. A unique ability of real-time quantitative polymerase chain reaction (qPCR) to determine gene copy number, even within a small percent of observed tumor cells, and can be more appropriate. *MYCN* genomic copy number from 44 human brain tumors (22 medulloblastomas and 22 neurocytomas) was determined by means of FISH, array-CGH, and qPCR. By qPCR, with the original set of oligonucleotides, 17 out of 44 (38.6%) tumors were found to contain a

1.3- to 2.9-fold increase of *MYCN* defined as low-level gain. An absolute qPCR method was used to get high accuracy of results. Strong correlation was observed between the three methods: for medulloblastomas, $r = 1$ ($P < 0.01$) between FISH and array-CGH and $r = 0.92$ ($P < 0.01$) between qPCR and FISH/array-CGH. For neurocytomas, $r = 0.9$ ($P < 0.01$) between FISH and array-CGH and $r = 0.34/0.43$ ($P < 0.01$) between qPCR and FISH/array-CGH. Absolute qPCR assays possess high precision compared to other conventional methods and can be used for accurate and quickness detection of *MYCN* status (low-level gene gain and amplification). *J. Clin. Lab. Anal.* 22:123–130, 2008. © 2008 Wiley-Liss, Inc.

Key words: gene amplification; low-level gain; medulloblastoma; neurocytoma; TBP

INTRODUCTION

MYCN was the first amplified oncogene that turned out to be of clinical significance due to its association with aggressively growing tumor phenotypes. The initial surveys suggested *MYCN* amplification was specific for neuroblastoma. Later studies revealed that it could be seen in some other tumors (1) and appeared to serve as indicator for neuroblastoma poor prognosis (2). *MYCN* expression is essential for the rapid expansion of neural progenitor cells during central nervous system development (3,4) and inhibition of neuronal differentiation; therefore, it plays a significant role in neurogenesis. In medulloblastomas, *MYCN* amplification is rare (1,5), while overexpression is frequently observed (6). Amplification of the *MYCN* oncogene in neurocytoma analyzed by polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) methods either was not revealed (7) or it was rare (8). In a recent investigation, the gain of *MYCN* protooncogene in 70% of neurocytomas was

found using array-based comparative genomic hybridization (array-CGH) (9). *MYCN* is supposed to be a “driver oncogene” in neurocytoma pathogenesis and aid in renewal of the tumor cells population.

The phenomenon of gene copy excess below the amplification threshold (3- to 10-fold) is defined as *MYCN* gain. This effect represents rearrangements at 2p24, such as duplications (10), or unbalanced translocations, frequently observed on advanced disease

*Correspondence to: Sofia Malakho, Engelhardt Institute of Molecular Biology RAS, Vavilov str., 32, Moscow, 119991, Russia. E-mail: _sophie@rambler.ru

Grant sponsor: Russian Foundation for Basic Research; Grant number: 04-04-48154; Grant sponsor: Presidential Program on Supporting the Leading Russian Schools in Science; Grant number: 1227.2006.4.

Received 25 June 2007; Accepted 12 December 2007

DOI 10.1002/jcla.20232

Published online in Wiley InterScience (www.interscience.wiley.com).

stages (3,11). Since low-level gain could hardly be quantified by existing traditional methods (12) (FISH, Southern blotting, and array-CGH), other techniques should be developed for its detection.

The real-time quantitative PCR (qPCR) method was used for determination of *MYCN* gain/amplification level in medulloblastomas and neurocytomas. Despite its obvious advantages, to our knowledge, this method was never used for such purposes in clinical diagnostics.

A new oligonucleotide set was designed for rapid and accurate measurement of *MYCN* gene dosages based on the real-time PCR method. The absolute content of *MYCN* and *TBP* (control gene encoding TATA box binding protein) in tumor DNA samples was obtained in one plate by extrapolation on calibration curves generated with reference DNA and subsequent comparison of copy numbers. By means of this approach we quantified *MYCN* gene copy number (including low-level gain) in 22 medulloblastomas and 22 neurocytomas.

MATERIALS AND METHODS

Medulloblastoma (n = 22) and central neurocytoma (n = 22) tissue specimens were collected at Burdenko Neurosurgical Institute between November 2002 and December 2005. All patients were untreated before the operation. Specimens from each tumor were taken at the time of the initial operation and then immediately frozen in liquid nitrogen and stored at -70°C . Histological analysis was made to confirm the presence of tumor cells in all specimens. The median age of patients with medulloblastoma was 7.5 years (range, 2–16); nine of them were males and 13 were females. The median age of patients with neurocytoma was 29.7 years (range, 16–59); three of them were males and 19 were females. Normal reference human DNA (Perkin Elmer, Foster City, CA) was used as a control.

DNA isolation was performed according to the protocol supplied with the DNeasy tissue kit (Qiagen, Hilden, Germany). The amount of purified DNA was quantified on a spectrophotometer (ND-1000 Spectrophotometer; NanoDrop[®], Wilmington, DE).

FISH Analysis of the *MYCN* Gene

A two-color FISH assay was performed on 5- μm -thick sections using a commercial set of fluorochrome-labeled probes: centromere 2p11–q11 probe and locus-specific 2p22 (*MYCN*) probe (Vysis, Inc., Abbott Laboratories SA, Downers Grove, IL). After protease pretreatment of the slides and their fixation, the probes (10 μL per slide) were applied to the sections. Simultaneous probe/specimen denaturation at 76°C for 8 min with subsequent overnight incubation at 37°C was performed in the HYBriteTM hybridization chamber

(Vysis, Inc., Abbott Laboratories SA). After the slide was washed with formamide, saline-sodium citrate (SSC), and Triton, the nuclei were counterstained with 4',6'-diamino-2-phenylindole (DAPI) (1,000 ng DAPI/mL in antifade mounting solution; Vysis Inc., Abbott Laboratories SA). The sections were studied with an Axioplan 2 fluorescent microscope (Karl Zeiss, Göttingen, Germany) that was equipped with a set of the appropriate filters (Vysis Inc., Abbott Laboratories SA). Signals were scored in at least 200 nonoverlapping, intact nuclei. Nonneoplastic temporal lobe specimens (n = 8) were used as a control for each probe pair. Specimens were considered amplified for *MYCN* when more than 10% of tumor cells exhibited either *MYCN*/CEP2 ratio >2 or innumerable tight clusters of signals of the locus probe. Polisomies (chromosomal gains) were defined as $>10\%$ of nuclei containing three or more signals for locus or CEP, as no such findings were found in the control brain tissue. Images were captured using a high-resolution CCD microscopy camera AxioCam MRm REV2 (Karl Zeiss, Göttingen, Germany). The resulting images were reconstructed with green (fluorescein isothiocyanate [FITC]), red (Cy3), and blue (DAPI) pseudocolors using the AxioVision 4 multichannel fluorescence basic workstation (Karl Zeiss) according to the manufacturer's instructions. A more detailed protocol was described previously (13).

Array-CGH

Approximately 100 ng of DNA from tumor samples (test DNA) and normal reference human DNA were labeled by a random priming reaction with Cy3-dCTP and Cy5-dCTP (Perkin Elmer, Foster City, CA), respectively, according to the protocols recommended by the manufacturer (Vysis, Inc., Abbott Laboratories S.A., Downers Grove, IL). DNA was denatured with $\times 2.5$ random priming solution at 100°C for 10 min, mixed with the nucleotide GenoSensor mix, fluorescent nucleotides, and Klenow fragment, and incubated at 37°C for 2 hr. After DNase treatment, unincorporated fluorescent nucleotides were removed by ethanol precipitation.

Aliquots of labeled DNA were mixed with the hybridization buffer, denatured at 80°C for 10 min, and incubated at 37°C for 1 hr to allow blocking of repetitive sequences. The hybridization mixture was then introduced on genomic DNA microarray slides. We used the commercially available GenoSensor Array 300 (Vysis, Inc., Abbott Laboratories SA) contained genomic targets which were cloned from P1 and BAC libraries. The microarray slides were incubated 66–72 hr at 37°C . The posthybridization process included subsequent washing in 50% formamide/ $2\times$ standard saline citrate solution (three times for 10 min each at 40°C) and

1 × standard saline citrate solution (four times for 5 min at room temperature). The microarray slides were counterstained with DAPI solution. Images were analyzed with the GenoSensor Reader software. After background subtraction, the program calculated total intensity and intensity ratio of test and reference signals for each target and indicated the degree of gain and loss copy number when compared with the sample's modal copy number. We determined gain and amplification of target DNA sequence copy numbers by a green/red ratio > 1.2, and > 2.0, respectively. A more detailed protocol was described previously (14).

Real-Time qPCR

Real-time qPCR was performed with the ABI Prism 7000 Sequence Detector System (Applied Biosystems, Foster City, CA). qPCR was carried out in a 25-μL reaction volume composed of: RealityTM universal PCR mix (Biochip-EIMB, Moscow, Russia) (15) with specific primers, probe and 5 μL of a 25–500 ng of template DNA. The sequences of the original primers and TaqMan probes labeled with FAM[®] and TAM-RA[®] (Molecular Probes, Carlsbad, CA) were as follows (Table 1). The final optimized concentration of each primer and TaqMan probe was 200 nM.

Thermal cycling conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C. One plate contained two sets of measurements: for target and control genes (for accuracy rating increasing and for reaction conditions unification). Five points in five replicates of plasmid DNA serial dilutions, a no template control, unknown and control (normal) samples were present in each set. The threshold cycles (Ct) were determined manually. Reference evaluations for each DNA sample were performed according to the endogenous control gene *TBP* encoding TATA-box binding protein. The absolute target copy number was calculated using a standard calibration curve generated from PCR reactions with serially diluted plasmids (pGEM-T vector; Promega, Madison, WI) containing the respective sequence verified inserts (10-fold dilutions from 30⁵ to 30 copies). All results were expressed as arbitrary units, representing the ratio of mean of *MYCN* copy number/mean of copy numbers of *TBP* target (normalized copy number, NCN). All the experiment lots were carried out in the minimal time period. Amplicons received from PCR reaction for each target gene were purified then sequenced (for normality verification).

Statistical Analysis

qPCR interrun variability and intersample variability were assessed by variance of controls among the plates

TABLE 1. Primers and TaqMan probes for real-time PCR

Gene, target	Primers sequence 5'-3' and position in genome DNA		TaqMan probe sequence 5'-3' position in genome DNA
	Forward	Reverse	
<i>TBP</i> , genome	TTAGCTGGCTCTGAGTATGAATAAC Intron (34444–34469)	GCTGGAACCACTTCTG Exon 6 (34536–34556)	CAGTCCAGACTGGCAGCAAGAAAAAT Exon 6 (34497–34522)
<i>MYCN</i> , genome	TGATGGACCCATAAAATAGCAGTC Intron (70704–70729)	TCTTGGGACGCACAGTGATG Exon 3 (70942–70962)	CTTCTCCACAGTGACACGTGATTCT Exon 3 (70872–70900)
<i>ACTB</i> , genome	GTGCTCAGGGCTTCTTGTCCTTT Intron (171127–171150)	TTTCTCCATGTCGTCACAGTTGGT Intron (171264–171288)	AAGGATTCTATGTGGCGACGAGGCCCA Intron (171183–171212)

TABLE 2. Alterations of *MYCN* gene in genome by three methods in medulloblastomas*

Sample number	qPCR, NCN	Array-CGH	FISH
1m	0.27	Balanced	Balanced
2m	0.37	Balanced	Balanced
3m	0.40	Balanced	Balanced
4m	0.41	Balanced	Balanced
5m	0.41	Balanced	Balanced
6m	0.48	Balanced	Balanced
7m	0.62	Balanced	Balanced
8m ^a	0.74	Amplified	Amplified
9m	0.86	Balanced	Balanced
10m	0.88	Balanced	Balanced
11m	0.89	Balanced	Balanced
12m	1.01	Gained	Gained
13m	1.04	Gained	Gained
14m	1.14	Balanced	Balanced
15m	1.46	Gained	Gained
16m	1.50	Gained	Gained
17m	1.67	Gained	Gained
18m	1.79	Gained	Gained
19m	2.11	Gained	Gained
20m	6.78	Amplified	Amplified
21m	11.57	Amplified	Amplified
22m	52.34	Amplified	Amplified

*All the samples are arranged according to real-time PCR data of *MYCN* gene copy number increase in genome. Samples that contained *MYCN* gene gain or amplification are bold.

^aCase with gained 6q24-27 region.

using Microsoft Excel (Microsoft, Redmond, WA) computer software (a ratio of NCN/mean copy number of controls in sets of plates). Associations between gene copy number increase of *MYCN* and other variable data were described by Pearson correlation coefficients (r , $P < 0.01$). Sampling was assumed to have normal distribution.

RESULTS

Lacking a commercial set for *MYCN* gene copy number quantification, oligonucleotide detection systems for *MYCN* and *TBP* genes were designed anew. The TaqMan probe and reverse primer are located in the exon, while the reverse primer has an intron position and distinguishes genome gene variant. All the oligonucleotides have acceptable GC-nucleotide composition.

Efficiency and sensitivity of selected primers and TaqMan probes were preliminarily validated on four medulloblastoma DNA samples and a control. Tumor specimens were analyzed previously by the FISH method: three of them contained reliably high amplification levels of the *MYCN* gene, and one of them did not have any *MYCN* gene copy number gain. This fact was confirmed by the qPCR method. The value of *MYCN* gene NCN in normal genome DNA was 0.5.

TABLE 3. Alterations of *MYCN* gene in genome by three methods in central neurocytomas*

Sample number	qPCR, NCN	Array-CGH	FISH
1n	0.42	Balanced	Balanced
2n	0.58	Gained	Gained
3n	0.63	Gained	Gained
4n	0.92	Balanced	Balanced
5n	1.07	Balanced	Gained
6n	1.14	Gained	Gained
7n	1.18	Gained	Gained
8n	1.22	Balanced	Balanced
9n	1.26	Balanced	Balanced
10n	1.28	Balanced	Balanced
11n	1.31	Gained	Gained
12n	1.37	Gained	Gained
13n	1.37	Gained	Gained
14n	1.43	Gained	Gained
15n	1.44	Balanced	Balanced
16n	1.53	Balanced	Balanced
17n	1.59	Gained	Gained
18n	1.62	Gained	Gained
19n	1.72	Gained	Gained
20n	1.95	Gained	Gained
21n	2.42	Gained	Gained
22n	2.89	Gained	Gained

*All the samples are arranged according to real-time PCR data of *MYCN* gene copy number increase in genome. Samples contained *MYCN* gene gain are bold.

In a tumor DNA sample (Table 2, sample 2 m) without amplification (FISH and array-CGH data), NCN of target gene in genome was the same as in control (0.37). In tumor samples with amplified *MYCN* NCN of the gene was greatly higher than in control (6.78, 11.18, and 52.34; see samples 20 m, 21 m, and 22 m in Table 2). These data correspond well with each other and demonstrate high specificity of new oligonucleotide sets for *MYCN* measurement by qPCR.

To choose the best control gene of genome stability we compared two genes—*TBP* and *ACTB* (encoding beta-actin). After the first experiments, the *TBP* gene was chosen as more suitable for further work because of its single copy in genome. Efficiency of PCR amplification (E) for the *TBP* gene (genome variant mean E = 95%) was comparable to *MYCN* gene efficiency (genome variant mean E = 97%), which is promising for their further correct comparison. Efficiency of PCR amplification for *ACTB* was noticeably higher (E = 99%) than that for the *MYCN* gene.

Altogether, *MYCN* gene copy number alterations were determined in 22 medulloblastomas and 22 central neurocytomas (Tables 2 and 3, respectively). For measurement reliability three detection methods were used: FISH (Fig. 1), array-CGH (Fig. 2), and real-time qPCR (Fig. 3). Summary data (without controls) are presented in Tables 2 and 3.

Fig. 1. *MYCN* gene different amplification rates by FISH analysis. Red-colored probe: *MYCN* region. Green signals: CEP-2. Typical “balanced” (normal) genome (A), *MYCN* gene low-level gain (B), and *MYCN* gene high amplification level (single red signals and “clouds”) (C).

Fig. 2. Profiles of genomic aberrations in medulloblastomas and central neurocytomas determined by array-CGH. **A:** Medulloblastoma genome profile with high *MYCN* gene amplification and typical isochromosome 17. **B:** Case of neurocytoma with *MYCN* gene low-level gain and with gains of 10q, 18q, and losses in chr 17 typical for this tumor type.

Gene amplification levels obtained for medulloblastomas by three methods are close to each other (Table 2); for example, $r = 0.92$ ($P < 0.01$) between qPCR and array-CGH data and $r = 1$ ($P < 0.01$) between array-CGH and FISH methods. Based on control DNA samples' distribution in all qPCR experiment sets, NCN value equal to 1.3 was taken as “cutoff” that divides normal samples (below) from tumors with extra gene copy number (beyond) (Fig. 3).

Among medulloblastomas, only eight tumors contained extra *MYCN* gene copies ($NCN = 1.46\text{--}52.34$), while in 14 samples *MYCN* gene quantity was within normal levels ($NCN = 0.27\text{--}1.14$). A total of two false-negative by qPCR samples (12m and 13m in Table 2) were found to contain *MYCN* low-level gain according to FISH and array-CGH methods. In one medulloblastoma (Table 2, sample 8m) that contained reliable high *MYCN* amplification by the array-CGH

Fig. 3. *MYCN* gene low-level gain profile in medulloblastoma and neurocytoma DNA samples by real-time PCR. NCN, normalized copy number (*MYCN/TBP*). White spots represent tumor samples, black spots represent control DNA samples. Dotted line of threshold (NCN = 1.3) divides all the samples into two groups: without any *MYCN* gene gain (NCN < 1.3) and with low-level gain (NCN > 1.3). All the control DNA samples are located beyond this threshold.

method, no copy number increase was obtained by qPCR. After further inquiry it turned out that only this sample also contains gain of *TBP* gene (6q24-27 region) used as a control in qPCR, which led to false data using NCN calculation. Although 6q aberrations are rare in tumors studied, this fact must be always taken into consideration.

Low-level gene copy number alterations in neurocytoma were hardly detectable by FISH and array-CGH and could be presented in “yes/no” data format. According to qPCR data (Fig. 3), nearly half of neurocytoma samples ($n = 12$) contained low-level gene gain (1.31- to 2.89-fold gene increase); this data was confirmed by FISH and array-CGH for 10 of the tumors. A total of six samples had just a slightly higher level of *MYCN* quantity than the control (NCN = 1.07–1.28; samples 5n–10n in Table 3). This level did not allow us to conclude that *MYCN* gene copy is increased here. The remaining four neurocytomas (1n–4n) have balanced gene quantity (NCN = 0.42–0.92) by qPCR. In samples 2n and 3n, *MYCN* low-level gain was detected by FISH and array-CGH.

Differences between data obtained by various methods could mainly belong to a baffling complexity of low-level gain detection. The qPCR method with our oligonucleotide set represents a rapid and inexpensive

tool for *MYCN* gene quantity measurement that is highly concordant with other accepted approaches.

DISCUSSION

Fast and accurate assessment of *MYCN* gene copy number is of the utmost importance for neuroblastoma tumor prognosis. Nevertheless, the use of two independent methods to determine *MYCN* status is recommended: Southern hybridization or dot-blot and FISH techniques (16). However, reliable identification of gene low-level gain with Southern blotting by comparison of band intensities is sometimes difficult. Clinical significance of *MYCN* low-level gain is not yet known, but it may be of research significance, especially for neurogenic tumors. Despite of the striking histobiological differences between neurocytoma and neuroblastoma, recurrent genetic alterations share some similarities in both tumors (9).

We validated sensitivity and specificity of our *MYCN* TaqMan detection system on histologically different tumors of neurogenic origin with previous diagnosis and available FISH and array-CGH data. All highly amplified and nonamplified tumors (medulloblastomas, $n = 4$) were detected by qPCR, and no false-positives was observed. By all three methods, eight out of 22

medulloblastoma DNA samples were found to contain *MYCN* gene gain. Among them, there were three samples with high *MYCN* amplification, while others ($n = 5$) contained gene low-level gain. As mentioned above, the *TBP* gene locus also can undergo some aberrations. This effect may influence final qPCR result. Other investigators prefer to use the control gene *ACTB* (17), while in our work it was less reliable than the *TBP* gene because of its differing amplification efficiency. This problem may be solved by using two or more control single-copy genes on various chromosomes with the same amplification efficiency value.

We compared the results obtained by the real-time PCR method with those obtained by conventional FISH and array-CGH in 22 cases of central neurocytoma. A total of 12 of the investigated tumors showed only a few additional *MYCN* copies defined as *MYCN* gain. These results were compared with FISH and array-CGH data for the same tumors. A total of two false-positive and four false-negative results were observed in qPCR data. Such disagreement in results is likely due to using of different parts of specimen with varied number of cells contained 2p24 aberrations. In contrast to the qPCR method, FISH and array-CGH data format do not allow a quantitative result. Therefore, it is difficult to discriminate different *MYCN* status correctly.

The Pearson correlation coefficient in group of medulloblastoma was valued as high as $r = 1$ ($P < 0.01$) between FISH and array-CGH and $r = 0.92$ ($P < 0.01$) between qPCR and FISH/array-CGH, thus displaying strong correlation between these three methods. Variation of $r = 0.9$ ($P < 0.01$) and $r = 0.34/0.43$ ($P < 0.01$) for FISH and array-CGH between qPCR and FISH/array-CGH is likely due to tissue heterogeneity of neurocytoma. Such tumors, in contrast to genetically homogeneous medulloblastomas, contain different numbers of cells with *MYCN* gain. When the detection results are more precise, then the ratio of tumor cells to normal cells in the specimen is higher.

Traditionally, three methods are used in modern molecular diagnostics: Southern or dot-blot, FISH, and array-CGH. FISH is the common "gold standard" and currently it is the only technology that avoids the problem of tumor-cell heterogeneity (3). This method is time-consuming and could hardly detect low-level gain because of the possible existing aneuploidy. Array-CGH allows the detection of single copy number changes (18,19). However, it is more expensive, time-consuming, and requires special equipment with software. FISH and array-CGH are not as sensitive as a real-time qPCR assay. The sensitivity of qPCR goes down as far as 10^{-4} – 10^{-5} (20) while the FISH sensitivity ranges between 1 to 3×10^{-2} (21). qPCR is less expensive, fast, and informative due to a large dynamic range of quantifica-

tion, no requirement for post-PCR sample handling, and the need for very small amounts of starting material (16,17,22–24). In addition, our results indicate that correlations between qPCR, FISH, and array-CGH data are strong (total $r = 0.97/0.76/0.74$, $P < 0.01$ between FISH/array-CGH, array-CGH/qPCR, and FISH/qPCR, respectively). This is in agreement with other similar studies (18,25,26).

For reliable detection of low-level copies using real-time PCR and further sample comparison, it is necessary to make an absolute quantification assay. Measurement of target and control genes in one reaction plate makes possible more precise results and comparative sample analysis. We have demonstrated that qPCR is the only accurate technique that can be applied both for amplification measurement (13,17,26) and for small amounts of DNA detection allowing rapid estimation of *MYCN* gene copy number. This is the only method that can deliver a quantitative result in 1 day. One might suppose that since in medulloblastomas and neurocytomas *MYCN* gain could be quantified by qPCR this could be also made for neuroblastoma.

Our study confirms that qPCR is an essential supplement to current technologies, e.g., FISH and array-CGH, but it cannot replace them. Accurate and fast quantitative detection of low-level gene alterations is possible only by means of absolute qPCR.

ACKNOWLEDGMENTS

We thank L.L. Kiselev for critical reading of this manuscript, A.I. Karseladze for kind assistance at the initial stage of this work, and A.S. Bavykin for helpful advice.

REFERENCES

1. Fruhwald MC, O'Dorisio MS, Rush LJ, et al. Gene amplification in PNETs/medulloblastomas: mapping of a novel amplified gene within the *MYCN* amplicon. *J Med Genet* 2000;37:501–509.
2. Schwab M, Corvi R, Amler L. N-MYC Oncogene amplification: a consequence of genomic instability in human neuroblastoma. *The Neuroscientist* 1995;1:277–285.
3. Spitz R, Hero B, Skowron M, Ernestus K, Berthold F. *MYCN*-status in neuroblastoma: characteristics of tumours showing amplification, gain, and non-amplification. *Eur J Cancer* 2004;40:2753–2759.
4. Knoepfler PS, Cheng PF, Eisenman RN. N-MYC is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. *Genes Dev* 2002;16:2699–2712.
5. Bayani J, Zielenska M, Marrano P, et al. Molecular cytogenetic analysis of medulloblastomas and supratentorial primitive neuroectodermal tumours by using conventional banding, comparative genomic hybridization, and spectral karyotyping. *J Neurosurg* 2000;93:437–448.

6. Kleihues P, Cavenee WK, editor. Tumors of the nervous system. Pathology and genetics: World Health Organization international classification of tumours. 2nd edition. Lyon, France: WHO/IARC; 2000. 314pp.
7. Jay V, Edwards V, Hoving E, et al. Central neurocytoma: morphological, flow cytometric, polymerase chain reaction, fluorescence in situ hybridization, and karyotypic analyses. *J Neurosurg* 1999;90:348–354.
8. Tong CY, Ng HK, Pang JC, Hu J, Hui AB, Poon WS. Central neurocytomas are genetically distinct from oligodendrogliomas and neuroblastomas. *Histopathology* 2000;37:160–165.
9. Korshunov A, Sycheva R, Golanov A. Recurrent cytogenetic aberrations in central neurocytomas and their biological relevance. *Acta Neuropathol* 2007;113:303–312.
10. Corvi R, Savelyeva L, Schwab M. Duplication of N-MYC at its resident site 2p24 may be a mechanism of activation in human neuroblastoma cells. *Cancer Res* 1995;55:3471–3474.
11. Schwab M, Westermann F, Hero B, Berthold F. Neuroblastoma: biology and molecular and chromosomal pathology. *Lancet Oncol* 2003;4:472–480.
12. Tanaka S, Tajiri T, Noguchi S, et al. Clinical significance of a highly sensitive analysis for gene dosage and the expression level of *MYCN* in neuroblastoma. *J Pediatr Surg* 2004;39:63–68.
13. Korshunov A, Sycheva R, Gorelyshev S, Golanov A. Clinical utility of fluorescence in situ hybridization (FISH) in non-brainstem glioblastomas of childhood. *Mod Pathol* 2005;18:1258–1263.
14. Korshunov A, Sycheva R, Golanov A. Genetically distinct and clinically relevant subtypes of glioblastoma defined by array-based comparative genomic hybridization (array-CGH). *Acta Neuropathol* 2006;111:465–474.
15. Manzeniuk OIu, Malakho SG, Pekhov VM, Kosorukova IS, Poltarauk AB. Characterization of the universal Russian reagent sets for real-time PCR and its application for molecular oncodiagnostic. *Mol Biol (Mosk)* 2006;40:349–356.
16. Tajiri T, Tanaka S, Shono K, et al. Quick quantitative analysis of gene dosages associated with prognosis in neuroblastoma. *Cancer Lett* 2001;166:89–94.
17. Raggi CC, Bagnoni ML, Tonini GT, et al. Real-time quantitative PCR for the measurement of *MYCN* amplification in human neuroblastoma with the TaqMan detection system. *Clin Chem* 1999;45:1918–1924.
18. Chen Q-R, Bilke S, Wei JS, et al. cDNA array-CGH profiling identifies genomic alterations specific to stage and *MYCN*-amplification in neuroblastoma. *BMC Genomics* 2004;5:70.
19. Pinkel D, Seagraves R, Sudar D, et al. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. *Nat Genet* 1998;20:207–211.
20. Gabert J, Beillard E, van der Velden VHJ, et al. Standardization and quality control studies of “real time” quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia. A Europe Against Cancer Program. *Leukemia* 2003;17:2318–2357.
21. Perez-Simon JA, Caballero MD, Hernandez-Rivas JM, Chillón C, García-Isidoro M, San Miguel JF. Complete cytogenetic but not molecular remission in a patient with myeloid blast crisis of chronic myeloid leukemia treated with carboplatin and ARA-C. *Haematologica* 1997;82:604–605.
22. De Preter K, Speleman F, Combaret V, et al. Quantification of *MYCN*, *DDX1*, and *NAG* gene copy number in neuroblastoma using a real-time quantitative PCR assay. *Mod Pathol* 2002;15:159–166.
23. Gallego S, Reventos J, Detolledo JS, Munell F. Differential polymerase-chain reaction with serial dilutions for quantification of *MYCN* gene amplification in neuroblastoma. *Anticancer Res* 1998;18:1211–1215.
24. Boensch M, Oberthuer A, Fischer M, et al. Quantitative real-time PCR for quick simultaneous determination of therapy-stratifying markers *MYCN* amplification, deletion 1p and 11q. *Diagn Mol Pathol* 2005;14:177–182.
25. Dallas PB, Gottardo NG, Firth MJ, et al. Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT-PCR—how well do they correlate? *BMC Genomics* 2005;6:59.
26. Anderson J, Gibson S, Williamson D, et al. Rapid and accurate determination of *MYCN* copy number and 1p deletion in neuroblastoma by quantitative PCR. *Pediatr Blood Cancer* 2006;46:820–824.