**MYCN is amplified during S phase, and c-myb is involved in controlling MYCN expression and amplification in MYCN-amplified neuroblastoma cell lines**

NEVIM AYGUN and OGUZ ALTUNGOZ

Department of Medical Biology, Faculty of Medicine, Dokuz Eylul University, Izmir 35340, Turkey

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**Abstract.** Neuroblastoma is a common type of solid tumor in infants. **MYCN proto-oncogene bHLH transcription factor (MYCN)** amplification and 1p36 deletion are important factors associated with the poor prognosis of neuroblastoma. Expression levels of **MYCN** and **c-MYB proto-oncogene transcription factor (c-myb)** decline during the differentiation of neuroblastoma cells; E2F transcription factor 1 (E2F1) activates the **MYCN** promoter. However, the underlying mechanism of **MYCN** overexpression and amplification requires further investigation. In the present study, potential c-Myb target genes, and the effect of c-myb RNA interference (RNAi) on **MYCN** expression and amplification were investigated in MYCN-amplified neuroblastoma cell lines. The mRNA expression levels and **MYCN** gene copy number in five neuroblastoma cell lines were determined by quantitative polymerase chain reaction. In addition, variations in potential target gene expression and **MYCN** gene copy number between pre- and post-c-myb RNAi treatment groups in **MYCN**-amplified Kelly, IMR32, SIMA and MHH-NB-11 cell lines, normalized to those of non-MYCN-amplified SH-SY5Y, were examined. To determine the associations between gene expression levels and chromosomal aberrations, **MYCN** amplification and 1p36 alterations in interphases/metaphases were analyzed using fluorescence in situ hybridization. Statistical analyses revealed correlations between 1p36 alterations and the expression of c-myb, **MYB proto-oncogene like 2 (B-myb)** and **cyclin dependent kinase inhibitor 1A (p21)**. Additionally, the results of the present study also demonstrated that c-myb may be associated with E2F1 and **L3MBTL1 histone methyl-lysine binding protein** (L3MBTL1) expression, and that E2F1 may contribute to **MYCN**, B-myb, p21 and chromatin licensing and DNA replication factor 1 (hCdt1) expression, but to the repression of **geminin** (GMNN). On c-myb RNAi treatment, L3MBTL1 expression was silenced, while GMNN was upregulated, indicating G1/M arrest. In addition, **MYCN** gene copy number increased following treatment with c-myb RNAi. Notably, the present study also reported a 43.545% sequence identity between upstream of **MYCN** and *Drosophila melanogaster* amplification control element 3, suggesting that expression and/or amplification mechanisms of developmentally-regulated genes may be evolutionarily conserved. In conclusion, c-myb may be associated with regulating **MYCN** expression and amplification. c-myb, B-myb and p21 may also serve a role against chromosome 1p aberrations. Together, it was concluded that **MYCN** gene is amplified during S phase, potentially via a replication-based mechanism.

**Introduction**

**MYCN proto-oncogene bHLH transcription factor (MYCN)** amplification and 1p36 deletion are important factors associated with poor prognosis in neuroblastoma (1-3), one of the most types of infant malignancy (4). **MYCN** amplification, which leads to **MYCN** overexpression, has been reported in 18-38% of cases of neuroblastoma and in a panel of neuroblastoma cell lines (3,5-9). As a developmentally-regulated gene, **MYCN** is highly expressed in dorsal root ganglia, sympathetic chain ganglia and the spinal cord in the human fetus during the development of the sympathetic nervous system at 8.5 weeks of gestation (9). In addition, the chromosome 1p36 locus is frequently deleted in neuroblastoma cell lines (10). **Chromodomain helicase DNA binding protein 5, calmodulin binding transcription activator 1, kinesin family member 1B, castor zinc finger 1 and microRNA (miR)-34a** have been analyzed as the strongest candidate tumor suppressor genes at the 1p36 locus in neuroblastoma (11,12).

**c-MYB** proto-oncogene transcription factor (c-Myb) has been reported to be associated with cell growth and proliferation in neuroblastoma (13). On induction by retinoic acid, **cyclin dependent kinase inhibitor 1A (p21)** increases, mediating the G1 arrest initiated by the expression of **p21** in vivo. The central role of **p21** in cell cycle regulation is associated with poor prognosis in neuroblastoma (14). **MYCN** proto-oncogene bHLH transcription factor (MYCN) amplification and 1p36 deletion are important factors associated with the poor prognosis of neuroblastoma. Expression levels of MYCN and c-MYB proto-oncogene transcription factor (c-myb) decline during the differentiation of neuroblastoma cells; E2F transcription factor 1 (E2F1) activates the MYCN promoter. However, the underlying mechanism of MYCN overexpression and amplification requires further investigation. In the present study, potential c-Myb target genes, and the effect of c-myb RNA interference (RNAi) on MYCN expression and amplification were investigated in MYCN-amplified neuroblastoma cell lines. The mRNA expression levels and MYCN gene copy number in five neuroblastoma cell lines were determined by quantitative polymerase chain reaction. In addition, variations in potential target gene expression and MYCN gene copy number between pre- and post-c-myb RNAi treatment groups in MYCN-amplified Kelly, IMR32, SIMA and MHH-NB-11 cell lines, normalized to those of non-MYCN-amplified SH-SY5Y, were examined. To determine the associations between gene expression levels and chromosomal aberrations, MYCN amplification and 1p36 alterations in interphases/metaphases were analyzed using fluorescence in situ hybridization. Statistical analyses revealed correlations between 1p36 alterations and the expression of c-myb, MYB proto-oncogene like 2 (B-myb) and cyclin dependent kinase inhibitor 1A (p21).

**Key words:** neuroblastoma, MYCN proto-oncogene bHLH transcription factor amplification, 1p36 deletion, c-MYB proto-oncogene transcription factor, E2F transcription factor 1, L3MBTL1 histone methyl-lysine binding protein, geminin DNA replication inhibitor, MYB proto-oncogene like 2, p21/cyclin dependent kinase inhibitor 1A
acid, c-myb and MYCN expression levels decrease during the differentiation stage of neuroblastoma cells (14-18). In humans, c-myb, MYB proto-oncogene like 2 (B-myb) and MYB proto-oncogene like 1 (A-myb) belong to the myb gene family, and contain highly-conserved N-terminal domains (19). The functional orthologs B-myb and Drosophila melanogaster-myb (Dm-myb) share essential conserved functions required for cell proliferation, whereas the paralogous genes, A-myb and c-myb, have acquired novel functions (20). The Dm-Myb protein complex is directly involved in DNA replication and binds to amplification-control-element-on-3 (ACE3) and replication origin-β in a site-specific manner (21), necessary for chordin gene amplification on the third chromosome in Drosophila ovarian follicle cells (22).

The Dm-myb complex regulates the expression of developmentally-regulated genes (23). Additionally, it has been proposed that this complex may be involved in the activation or repression of transcription and DNA replication, depending on the presence of E2F transcription factor 1 (E2F1) or E2F transcription factor 2 (E2F2) with other particular cofactors, respectively. Drosophila lethal (3) malignant brain tumor [D-L(3)mbt] protein has also been associated with the Myb-MuvB repressor complex (23).

The human homolog of D-l(3)mbt, L3MBTL1 histone methyl-lysine binding protein (L3MBTL1), is expressed in cancer cell lines and a variety of normal human tissues (24). L3MBTL1, a candidate tumor suppressor in del(20q12) myeloid disorders, is required for normal progression of the replication fork, and interacts with the minichromosome maintenance complex component 2-7, cell division cycle 45 and proliferating cell nuclear antigen components of the DNA replication machinery (25).

Chromatin licensing and DNA replication factor 1 (hCdt1) is an essential factor of the pre-replication complex and is inhibited by geminin (GMNN) protein to prevent re-replication during the S, G2 and M phases of the cell cycle (26). GMNN and hCdt1 are overexpressed in tumors and a variety of cancer-derived cell lines (27,29).

MYCN transcriptionally activates the p53 tumor suppressor gene to induce apoptosis (30); however, MYCN suppresses the cyclin dependent kinase inhibitor 1A (p21) gene, resulting in anti-apoptotic activity of neuroblastoma (31). MYCN overexpression sensitizes MYCN-amplified neuroblastoma cells to apoptosis via the induction of p53 (32). In addition, E2F-regulated B-myb and c-myb expression are elevated by apoptotic stimuli, causing neuronal death (33).

In the present study, potential c-Myb target genes, and the effect of c-myb RNA interference (RNAi) on MYCN expression and amplification in neuroblastoma were investigated. For this, a plasmid vector-mediated RNAi method with a short hairpin RNA (shRNA) directed against c-myb mRNA was used in MYCN-amplified neuroblastoma cell lines. The present study demonstrated that c-myb may induce the expression of E2F1 and L3MBTL1 and that E2F1 may be associated with the induction of MYCN, B-myb, p21 and hCdt1 expression, in addition to the repression of GMNN. In addition, the results demonstrated that MYCN gene copy number was increased following treatment with c-myb RNAi. These findings revealed that c-myb is involved in controlling MYCN expression and amplification in MYCN-amplified neuroblastoma cell lines. Following c-myb RNAi treatment, L3MBTL1 expression was completely silenced, whereas GMNN was upregulated; the results indicate G2/M arrest. Consequently, the present study demonstrated that the MYCN gene may be amplified during S phase, which may occur via a replication-based mechanism.

Materials and methods

Sequence comparison. The DNA sequences encompassing the D. melanogaster-ACE3 element and that upstream of human MYCN were compared using the LFASTAn alignment program (version 2; bioinfo.hku.hk/services/analyseq/cgi-bin/lfastan_in.pl). The DNA sequences of D. melanogaster-chordin gene cluster (GenBank accession no. X02497.1) and Homo sapiens-chromosome 2 genomic contig, including the MYCN gene (NCBI reference sequence NT_005334.16; region, 8493966-11135164) were downloaded from the NCBI website (ncbi.nlm.nih.gov).

Transcription factor binding site search. Transcription factor binding sites upstream (-1,021 to -143), including the enhancer and proximal promoter of MYCN gene were investigated using the TFSEARCH program (version 1.3; cbrc.jp/research/db/TFSEARCH.html). In addition, the location information of the regulatory transcription factor binding sites in the promoters of all genes investigated in the present study was obtained from Qiagen, Inc. (Valencia, CA, USA) as predicted by Text Mining Application (SABioscience Corporation; Qiagen, Inc.) and the University of California Santa Cruz (UCSC) Genome Browser (sabioscience.com/chipqpcrsearch.php?app=TFBS).

Cell culture. Kelly (no. ACC 355), IMR32 (no. ACC 165), SIMA (no. ACC 164), MHH-NB-11 (no. ACC 157) and SH-SY5Y (no. ACC 209) cell lines were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). Kelly, SIMA and MHH-NB-11 cells were cultured in RPMI-1640 (cat. no. FG1215; Biochrom AG; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (cat. no. S0113; FBS; Biochrom AG; Merck KGaA), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. In addition, the culture medium of MHH-NB-11 cells included 1X non-essential amino acids. IMR32 cells were cultured in RPMI-1640 (Biochrom AG; Merck KGaA) supplemented with 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1X non-essential amino acids. SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (cat. no. FG0415; DMEM; Biochrom AG; Merck KGaA), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2.

Fluorescence in situ hybridization (FISH). FISH was performed as previously described (3,34). In FISH experiments, MYCN gene (2p24)/Chromosome 2 α-Satellite (red/green; cat. no. PONC0224; Qbiogene, Inc.; Thermo Fisher Scientific, Inc., Waltham, MA, USA), Ip36/Chr 1 SE
Neuroblastoma cells were seeded into 25-cm² tissue culture flasks and grown in culture medium specific for each cell line (please see ‘Cell culture’ section for the characteristics of each growth medium) in a humidified atmosphere containing 5% CO₂ at 37°C. The cells were detached with trypsin-EDTA (Biochrom AG; Merck KGaA) and incubated with 80 μl colcemid (Biological Industries, Kibbutz Beit Haemek, Israel) in a tube containing 5 ml culture medium in a 37°C water bath for 30 min. Following centrifugation at 300 x g for 10 min at room temperature, the cell pellets were incubated with 8 ml hypotonic solution (0.075 M) (Biochrom AG; Merck KGaA) in a 37°C water bath for 30 min. For the fixation of the cell pellets, 5 ml fresh Carnoy’s solution (3:1 Methanol:Glacial Acetic Acid; Merck KGaA) was used for 2-3 min at room temperature followed by centrifugation at 300 x g for 10 min (repeated five times).

The homogenized cells were dropped onto slides, and a 2X SSC (AppliChem GmbH, Darmstadt, Germany)/0.5% NP-40 (AppliChem) mixture was used for washing the slides in a 37°C water bath for 30 min. The slides were dehydrated in an ethanol (AppliChem) series of 70, 85 and 96%, respectively. The double-stranded DNAs on the slides were denatured in 70% formamide (AppliChem)/2X SSC (AppliChem) at 70°C for 2 min (5 min for Abbott/Vysis 1p36 probe). The denaturation of probes was performed in a 96°C water bath for 5 min (5 and 10 min at 75°C for Vysis and Poseidon 1p36 probes, respectively). The DNAs were hybridized with the probes via overnight incubation at 37°C in a hybridization box. The 0.5X SSC (AppliChem)/0.1% SDS (Honeywell Riedel-de Haën AG, Seelze, Germany), 1X PBD including NP40 (AppliChem) and Tween 20 (Santacruz Biotechnology, Inc., Dallas, Texas, USA) and 70% ethanol (AppliChem), respectively, were used for washing the slides after hybridization overnight. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole II (DAPI II; cat. no. 30-804841; Abbott Pharmaceutical Co. Ltd.) suspended in an antifade solution.

FISH slides were analyzed under an epifluorescence microscope (magnification, x100; Nikon Eclipse E600, Nikon Corporation, Tokyo, Japan) equipped with DAPI, FITC, rhodamine and triple band-pass filter sets. FISH images from interphase nuclei and metaphase spreads were captured using a high-sensitivity monochrome charge-coupled device camera, which was integrated with a Macintosh computer and processed with MacProbe imaging software (version 4.0, PSI Scientific Systems, League City, TX, USA).

In FISH analyses, 2 red and 2 green signals for MYCN and internal control per diploid genome were considered normal; 3-5 red signals vs. 2 green signals per diploid genome were scored as a low copy number of the MYCN gene; 6-10 red signals vs. 2 green signals per diploid genome were scored as an intermediate copy number of MYCN gene, whereas >10 red signals vs. 2 green signals per diploid genome indicate high copy number of MYCN.

For 1p36 and the internal control, 2 orange/red and 2 green signals per diploid genome were considered normal. Only 1 orange/red signal vs. ≥2 green signals per diploid genome was classified as a 1p36 deletion. A total of 2 orange/red signals vs. 3 green signals per diploid genome and so forth (representing at least one more signal number of control than that of the 1p36 probe) were considered to indicate an imbalance in 1p36 copy number; 3 orange/red and 3 green signals per diploid genome were classified as a 1p36-3/3 balanced alteration.

**DNA extraction.** Kelly, SIMA, IMR32, MHH-NB-11 and SH-SY5Y cells were seeded into 75-cm² tissue culture flasks and grown in a culture medium until cells reached near-confluence in a humidified atmosphere containing 5% CO₂ at 37°C. Genomic DNA was extracted using phenol:chloroform:isoamyl alcohol (25:24:1, respectively), pH 8.0 (cat. no. A0889.0500; AppliChem GmbH, Darmstadt, Germany) as previously described (35).

**cDNA synthesis.** Kelly, SIMA, IMR32, MHH-NB-11 and SH-SY5Y cells were seeded into 24-well plates at a density of 4x10⁴ cells in 0.75 ml culture medium per well and grown in a culture medium until cells reached near-confluence in a humidified atmosphere containing 5% CO₂ at 37°C. First-strand cDNAs were synthesized directly from adherent cultured cells, without requiring RNA purification or RNase H digestion steps, using the FastLane Cell cDNA kit (cat. no. 215011; Qiagen, Inc.) according to the manufacturer’s protocol. The procedure of this kit involves four steps: i) removing extracellular contaminants, ii) performing cell lysis and RNA stabilization, iii) eliminating genomic DNA and iv) producing the first-strand cDNA via reverse transcription. This kit has been optimized for use particularly in real-time, two-step reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The genomic DNA from the FastLane lysate was eliminated using gDNA Wipeout Buffer at 42°C for five min. The lysate was placed immediately on ice. The reverse-transcription reaction was performed at 42°C for 30 min; later reverse transcriptase enzyme was inactivated for finishing reaction at 95°C for three min according to the manufacturer’s protocol.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** In neuroblastoma cell lines, the expression levels of c-myb and potential target genes, and MYCN gene copy number compared with hypoxanthine phosphoribosyltransferase 1 (HPRT1) and p53 reference genes, respectively, were determined via qPCR on a LightCycler® 2.0 instrument (Roche Diagnostics, Basel, Switzerland). Relative quantitative qPCR experiments were performed using the LightCycler® TaqMan® Master kit (cat. no. 04535286001; Roche Applied Science, Penzberg, Germany) with prevalidated hydrolysis probes, Universal ProbeLibrary Set, Human (cat. no. 04683633001; Roche Applied Science) and primers according to the manufacturer’s protocol.

Probe and primer sets were designed using the web-based ProbeFinder software (version 2.40) via the UPL assay design center (www.universalprobelibrary.com). Primer pairs were...
Table I. Probe and primer pairs used for quantitative polymerase chain reaction.

A, mRNA

| NCBI accession number | Probe catalogue number | Right primer (5'‑3') | Left primer (5'‑3') | Amplicon size (nt) |
|-----------------------|------------------------|----------------------|---------------------|-------------------|
| A-myb BC101186.1      | 2, 04684982001          | aagaagttgctggcaga    | cttctttaagaatggtcctg | 75                |
| B-myb X13293.1        | 26, 04687574001         | gcagagaccttggcttct   | cccgagaagaaagagaaga | 68                |
| c-myb M15024.1        | 62, 04688619001         | agcttcgagtggcttct    | tgcctcaatgtcaacgaga | 72                |
| MYCN BC002712.2       | 55, 04688520001         | cctcttcatctcttttcag  | ccaagcggcctggaaga  | 68                |
| E2F1 BC050369.2       | 5, 04685024001          | egtgtaacccctctcaag   | tccaagacccacccagg  | 75                |
| E2F2 BC053676.1       | 68, 04688678001         | gctgtgacctaaacctcct  | ggacagcggacccaggaa | 93                |
| CDK2 BC003065.2       | 50, 04688112001         | cagatctccagggatagggg | cttcttcgcttacataa  | 104               |
| GMNN AF067855.1       | 53, 04688503001         | ecagagttgctaaaaaagc  | aacgtgacgaggtggaca | 72                |
| hCd1 AB053172.1       | 10, 04685091001         | agcaagttcttctctctcct | gcggagctgcttgccttc | 117               |
| L3MBTL1 BC039820.1    | 82, 04689054001         | ttcctttcttttttctcctc | aacgtgagcggatcagaga | 72                |
| p21 BC000275.1        | 70, 04688937001         | agcttgctgctgctctcc   | cggaggacactgaggag  | 112               |
| p53 AB082923.1        | 12, 04685113001         | cccctttgtgctctgtcctg | agcgctggacacgcaagg | 85                |
| p27 BC001971.1        | 1, 04684974001          | eggttacacttctgtggtc  | agatgtcacaagtgctgag | 130               |
| HPRT1 BC000578.2      | 73, 04688961001         | egacagcaagctcttccct  |tgaccttgatttggctcatac | 102               |

B, DNA

| NCBI accession number | Probe catalogue number | Right primer (5'‑3') | Left primer (5'‑3') | Amplicon size (nt) |
|-----------------------|------------------------|----------------------|---------------------|-------------------|
| MYCN Y00664.1         | 36, 04687949001        | ggccttaggctgcacaga   | tgacacagggctacaca  | 60                |
| p53 U94788.1          | 23, 04686977001        | gctagctcagcttctctct  | ttcgctgctgctctcctc | 88                |

B-myb, MYB proto‑oncogene like 2; A‑myb, MYB proto‑oncogene like 1; c‑Myb, transcriptional activator Myb; MYCN, MYCN proto‑oncogene bHLH transcription factor; E2F1, E2F transcription factor 1; E2F2, E2F transcription factor 2; CDK2, cyclin dependent kinase 2; GMNN, geminin; hCd1, chromatin licensing and DNA replication factor 1; L3MBTL1, L3MBTL1 histone methyl‑lysine binding protein; p21, cyclin‑dependent kinase inhibitor 1A; p27, cyclin‑dependent kinase inhibitor 1B; HPRT1, hypoxanthine phosphoribosyltransferase 1.

synthesized by Gene Link, Inc. (Hawthorne, NY, USA). NCBI accession numbers, probe/primer sets and amplicon sizes are presented in Table I.

In the calculation of both mRNA expression levels and gene copy number in neuroblastoma cell lines, the following equations (ΔΔCp method) were used: Presumed as E=2, ΔCp=(Cp_target−Cp_reference) and R2=ΔΔCp (36). A heatmap for visualizing gene expression and MYCN gene copy number within neuroblastoma cell lines was produced using Heatmapper software (www.heatmapper.ca) (37).

In addition, MYCN copy number and gene expression levels from MYCN-amplified neuroblastoma cell lines prior to- and post-treatment with c-myb RNAi as normalized to the Cp values of target and reference genes of SH-SY5Y cells, were determined using the following equation (ΔΔCp method): Presumed as E=2:

\[ R^2 = 2^{-\Delta\Delta C_p} = 2^{-[\Delta C_{p_{target}}(sample−control)−\Delta C_{p_{reference}}(sample−control)]} \] (36).

In the efficiency-corrected (dilution) method, to determine the slope (S), the standard curves were produced between mean Cp values and logarithms (logs) of five serial starting template concentrations (ranging from 2-32 ng) using Excel (v. 2; Turkish, Home and Student version/initial release date; 07/17/2007, Microsoft Corporation, Redmond, WA, USA). E and R2 values were calculated using the following equations:

\[ E_{target or ref} = 10^{\frac{\Delta \Delta C_p}{\Delta \Delta C_p_{(target−control)}}} \] and \[ R^2 = \frac{(E_{target or ref})^{\Delta \Delta C_p}_{(control−sample)}}{(E_{ref})^{\Delta \Delta C_p}_{(control−sample)}} \] (36,38).

RNAi. In the MYCN-amplified Kelly, SIMA, IMR32 and MHH-NB-11 cell lines, c-myb mRNA expression was dysregulated using a specific shRNA-expressing pre-made plasmid DNA vector (custom-made psiRNA-h7SKneo G1 kit; cat. no. siRNAa3-n21; InvivoGen, San Diego, CA, USA) according to the manufacturer's protocols.

A shRNA insert in the double-stranded RNA structure, which specifically targeted c-myb mRNA (NCBI accession no. M15024.1), was designed using InvivoGen siRNA wizard software (version 2.4; sirnawizard.com) and later ligated into the psiRNA-h7SKneo G1 expression vector. Pre-made plasmid DNA vectors (InvivoGen) expressing shRNAs for enhanced green fluorescent protein (EGFP) or irrelevant genes served as shRNA controls. Plasmid vectors containing the sequences of shRNA targeting c-myb mRNA and control shRNAs were transformed into E. coli Lysocomp GT116 strain (InvivoGen).

For SpeI enzyme digestion and DNA sequencing, plasmid DNA was extracted from transformed cells using the High
Pure Plasmid Isolation kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocols. Plasmid DNA vector expressing the shRNA directed against c-myb mRNA and EGFP control vector were cut with the _SpeI_ restriction enzyme (cat. no. ER1252; Thermo Fisher Scientific, Inc.) at a concentration of 10 U/µl (final concentration 0.5 U/µl) incubated in a 37°C water bath for 14 h and imaged following agarose gel electrophoresis (1%) stained with ethidium bromide. DNA Marker II was used for genomic DNA analysis (cat. no. SM0351; Fermentas; Thermo Fisher Scientific, Inc.). The shRNA sequences of c-myb and EGFP vectors were confirmed by plasmid DNA sequencing (Macrogen, Inc., Seoul, Korea) in both directions using forward (OL559) and reverse (OL408) primers. Primer sequences: OL559 primer (forward) 5'-CGATAAGTAACCTGAGCTAAGT-3' and OL408 primer (reverse) 5'-GCTTACTATGGGAACTAC-3', c-myb shRNA, oligo 1 (forward) 5'-ACCTCGGTATCTGCAGGATCTCTCATCAAGAGTGGAAGACTCTGGCAGATAACCTT-3' and oligo 2 (reverse) 5'-CCTGCTGGGTTACCTCTCTGGAGTAAAC-3'; EGFP shRNA, oligo 1 (forward) 5'-ACCCTGCAAGCCTGAGCTTACCTGTGAGGTTAGAAGCTCCTGCTGAGATAACCCG-3'; MYCN promoter, share a 43.545% sequence identity in an overlap of 914 nucleotides with a DNA fragment (bases 14 to 884) of the human MYCN gene (−1,021 to −143; bases 2,466,909 to 2,467,787), including the enhancer and proximal promoter, share a 43.545% sequence identity in an overlap of 914 nucleotides with a DNA fragment (bases 14 to 884) spanning the D. melanogaster ACE3 sequence. This result suggested that the expression and/or amplification mechanisms of developmentally-regulated genes may be conserved among various organisms during the evolutionary process.

**Statistical analysis.** Statistical differences were determined using a one-tailed Wilcoxon signed-rank test. For two-tailed analysis, Spearman's (r) and Pearson's (r) coefficients were used to investigate correlation; correlation coefficients (r, r) were evaluated according to the classified criteria as low (0.00-0.24), moderate (0.25-0.49), strong (0.50-0.74) and very strong (0.75-1.00) (39). P<0.05 was considered to indicate a statistically significant difference. All analyses were performed using SPSS software version 11.0 (SPSS, Inc., Chicago, IL, USA).

**Results**

**Sequence upstream of human MYCN gene shares ~44% sequence identity with a region encompassing ACE3, upstream of choriogenic genes in D. melanogaster.** It has been determined that promoter and enhancer regions are located ~200 and 800 bp upstream of MYCN, respectively, and have been reported to be responsible for basal and cell type-specific expression in a variety of murine and human cell lines (40). In addition, the similar organization of the origin elements and replicators, including ACE3 and DHFR, from _Drosophila_, mammals, _Tetrhydymena_ and _Sciara_ has been reviewed previously (41). In normal human cells, amplified DNA has not been observed at frequencies greater than 1/10^5, and MYCN expression has been reported to be limited in normal human adult tissues (42,43).

Taken together, previous reports have suggested that certain _trans_—acting and epigenetic factors may serve important roles in the transcriptional and/or epigenetic control of MYCN expression and amplification. It was hypothesized that the DNA sequences of upstream _cis_-regulatory elements of amplifiable genes in a variety of organisms may be evolutionarily conserved, at least in part. The present study thus investigated the degree of sequence similarity between sequence upstream of the human MYCN gene and the ACE3 element controlling chorion gene amplification in _D. melanogaster_ (44).

Initially, the DNA sequences encompassing MYCN upstream and ACE3 were compared using the LFASTAn program (Fig. 1A). The results revealed that sequences upstream of the human MYCN gene (~1,021 to ~143; bases 2,466,909 to 2,467,787), including the enhancer and proximal promoter, share a 43.545% sequence identity in an overlap of 914 nucleotides with a DNA fragment (bases 14 to 884) spanning the _D. melanogaster_ ACE3 sequence. This result suggested that the expression and/or amplification mechanisms of developmentally-regulated genes may be conserved among various organisms during the evolutionary process.

**Putative c-Myb and E2F1 binding sites detected in the enhancer and proximal promoter located upstream of MYCN.** Enhancer (~980 to ~860), inhibition (~860 to ~797) and promoter (~797 to ~108) regions of MYCN were previously identified in IMR32 cells (45). Additionally, MYCN expression mediated by this enhancer is induced at higher levels within IMR32 cells than in HeLa cells, indicating cell type-specific enhancement of MYCN expression. Recently, it was reported that the DNA binding motif of MYB (c-Myb) is specifically enriched in low-complexity transcription factor binding site (TFBS)-clustered regions (46), which suggested that c-Myb may contribute to cell type-specific transcriptional regulation.

To identify the putative transcription factor binding sites upstream of MYCN, the TFSEARCH program was employed (Fig. 1B). A total of two c-Myb and three E2F1 binding sites were identified in the enhancer and proximal promoter located upstream of MYCN, which shares partial sequence identity with a region encompassing ACE3. The bioinformatics data analysis of the present study indicated that c-Myb and E2F1 may be involved in the expression of the MYCN gene in neuroblastoma cells.

**MYCN amplification status, 1p36 alterations and ploidy level determined by FISH analysis in neuroblastoma cell lines.** To determine MYCN amplification status and 1p36 alterations in neuroblastoma cells (Fig. 2), interphase nuclei and metaphase spreads were analyzed using FISH. The degree of MYCN amplification (~10 copies per nucleus) was notably high in Kelly, SIMA, MHH-NB-11 and IMR32 cells (Table II); however, the fluorescence signal intensity of nuclei for each MYCN-amplified cell line differed (Fig. 2B). The SH-SY5Y cell line lacking MYCN amplification most commonly included three copies (93.1%) of MYCN (Table II and Fig. 2B). Our recent study demonstrated that three copies of MYCN in SH-SY5Y may be due to an unbalanced translocation involving the MYCN locus at 2p24 (34).
of metaphase spreads typically demonstrated homogeneously staining regions (HSRs) in Kelly, MHH-NB-11, and IMR32 cells, and double minutes in SIMA cells (Fig. 2C). In addition, the ploidy levels of all cell lines were determined (Fig. 2C).

In the present study, IMR32 exhibited the highest degree of 1p36 deletion, whereas the percentage was markedly low in other neuroblastoma cell lines (Table II and Fig. 2D). The 1p36 imbalance was higher in SIMA and MHH-NB-11 compared with SH-SYSY, Kelly and IMR32 cells (Table II and Fig. 2D). 1p36-3/3 balanced alterations were observed at a higher percentage in Kelly cells compared with other cell lines and were negatively correlated with 1p36 deletion (Table II and Fig. 2D).

Taken together, FISH analyses revealed that all the neuroblastoma cell lines analyzed in the present study contained aneuploid cell clones. Additionally, MYCN-amplified cell lines exhibited MYCN amplification and at least one 1p36 alteration.

Figure 1. Sequence comparison between MYCN upstream and ACE3, and transcription factor binding sites in MYCN upstream. (A) A 1,020 bp DNA sequence (top; bases 1-1,020) encompassing ACE3 from Drosophila melanogaster chorion gene cluster and a DNA sequence of ~65 kb (bottom) containing MYCN (bases 2,416,201 to 2,481,180) from human chromosome 2 genomic contig were compared using the LFASTAn program. (B) Using the TFSEARCH program, putative c-Myb and E2F1 binding sites upstream (-1,021 to -143) of human MYCN gene were detected. MYCN, MYCN proto-oncogene bHLH transcription factor; ACE3, amplification-control-element-on-3; c-Myb, transcriptional activator Myb; E2F1, E2F transcription factor.
as structural chromosome aberrations, whereas SH-SY5Y cells exhibited 1p36 imbalance, but no MYCN amplification.

c-myb expression, candidate target gene expression and MYCN gene copy number determined by qPCR in neuroblastoma cell lines. The candidate c-Myb target genes were selected using published literature on neuroblastoma tumor biology and gene amplification mechanisms, together with predicted binding site data for transcription factors in gene promoters (SABiosciences Text Mining Application and UCSC Genome Browser).

The present study investigated the associations between c-myb expression, candidate target gene expression, MYCN gene copy number and 1p36 alterations. MYCN gene copy number and the expression levels [MYCN, c-myb, B-myb, A-myb, E2F1, E2F2, p53, p21, cyclin-dependent kinase inhibitor 1B (p27), hCdt1, GMNN, cyclin dependent kinase 2 (CDK2) and L3MBTL1] were determined in neuroblastoma cell lines compared with the reference genes, p53 and HPRT1, via qPCR prior to c-myb RNAi (Table III and Fig. 3A). The expression levels of MYCN and B-myb were notably higher compared with those of E2F1, hCdt1, GMNN, p27, CDK2, p21, L3MBTL1 and c-myb in MYCN-amplified cell lines (Table III and Fig. 3B).

A-myb, E2F2 and p53 were not expressed in any of the five neuroblastoma cell lines (Table III). E2F2 has been analyzed as a candidate tumor suppressor gene located at the 1p36 locus, which is deleted in 25% of tumors and 87% of cell lines in neuroblastoma (2,10). According to FISH analysis, the 1p36 deletion was observed at a high percentage only in the IMR32 cell line (Table II), suggesting that E2F2 may be epigenetically repressed in cell lines lacking the 1p36 deletion.
Table II. *MYCN* amplification and 1p36 alterations in neuroblastoma cell lines.

| Cell line | MYCN amplification (%) | 1p36 alterations (%)<sup>b</sup> |
|-----------|------------------------|---------------------------------|
|           | Normal | Low | Intermediate | High | 2/2 | 3/3<sup>c</sup> | Deletion<sup>c</sup> | Imbalance |
| SH-SY5Y   | 3.8    | 96.2 | 0.0 | 0.0 | 67.1 | 2.8 | 0.4 | 28.4 |
| Kelly     | 0.0    | 0.0  | 0.0 | 100.0 | 2.4 | 49.8 | 0.1 | 11.4 |
| SIMA      | 0.0    | 1.5  | 3.4 | 95.1 | 7.4 | 0.9 | 0.4 | 91.1 |
| MHH-NB-11 | 0.0    | 0.0  | 0.0 | 100.0 | 0.4 | 3.4 | 0.0 | 95.7 |
| IMR32     | 0.0    | 0.0  | 0.0 | 100.0 | 5.0 | 0.6 | 85.9 | 8.5 |

<sup>a</sup>Percentage of *MYCN* amplification and 1p36 alterations from 260 and 500 interphases/metaphases using the fluorescence in situ hybridization method in cell lines, respectively, was determined. <sup>b</sup>1p36 alteration other than 3/3-balanced, deletion and imbalance is not presented. <sup>c</sup>Spearman’s correlation coefficient, 1p36-3/3 vs. 1p36 deletion ($r_s=-0.87, P=0.054$). *MYCN* amplification was characterized as normal (2), low (3-5), intermediate (6-10) and high (>10). *MYCN, MYCN proto-oncogene bHLH transcription factor.*

Table III. Expression of candidate c-Myb target genes and *MYCN* gene copy numbers in neuroblastoma cell lines.

| Gene | Cell line (Cp/R) |
|------|------------------|
|      | SH-SY5Y | Kelly | SIMA | MHH-NB-11 | IMR32 |
| MYCN<sup>b</sup> | 34.84±0.19/0.073 | 26.41±0.19/8.75 | 28.79±0.22/1.99 | 29.22±0.32/2.39 | 27.66±0.08/4.66 |
| B-myb | 28.94±0.27/4.38 | 27.19±0.26/5.10 | 28.07±0.91/3.27 | 27.82±0.05/4.17 |
| HPRT1 | 31.07±0.13/1.00 | 29.54±0.32/1.00 | 29.78±0.05/1.00 | 30.48±0.15/1.00 | 29.88±0.11/1.00 |
| E2F1 | 32.88±0.02/0.29 | 30.13±0.12/0.66 | 31.05±0.46/0.67 | 30.78±0.13/1.33 | 30.48±0.10/0.66 |
| hCdt1 | 31.97±0.14/0.54 | 29.95±0.36/0.75 | 30.35±0.46/0.67 | 32.96±0.31/0.18 | 30.84±0.14/0.51 |
| GMNN<sup>c</sup> | 33.09±0.08/0.25 | 29.37±0.23/1.13 | 30.40±0.36/0.67 | 31.89±0.02/0.020 | 35.40±0.01/0.006 |
| p21<sup>d</sup> | 33.64±0.38/0.17 | 29.97±0.51/0.74 | 32.97±0.46/0.67 | 32.39±0.20/0.16 | 31.92±0.30/0.38 | 30.73±0.41/0.55 |
| CDK2 | 32.32±0.21/0.42 | 30.89±0.70/0.39 | 32.42±0.55/0.16 | 32.31±0.46/0.28 | 31.12±0.12/0.42 |
| p21 | 34.87±0.07/0.072 | 32.35±0.56/0.14 | 35.40±0.02/0.020 | 37.86±0.22/0.006 | 32.26±0.09/0.19 |
| L3MBTL1 | 38.83±1.16/0.005 | 34.99±0.71/0.023 | 35.17±1.61/0.024 | 37.17±0.81/0.010 | 34.52±1.29/0.040 |
| c-Myb<sup>b</sup> | 37.54±0.85/0.011 | 34.59±0.84/0.030 | 37.18±1.08/0.006 | 37.18±0.20/0.010 | 36.11±0.12/0.013 |
| A-myb | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| E2F2 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| p53 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

<sup>a</sup>Data (mRNA) are expressed as mean Cp ± standard error of duplicate or triplicate independent experiments in case of discordant results. <sup>b</sup>Pearson’s correlation coefficients ($r_s=0.88, P<0.05$; $r_s=0.86, P=0.059$), respectively. 

<sup>c</sup>The *MYCN* DNA copy number of *MYCN*-amplified cell lines was calculated from mean Cp ± standard error of the mean of ≥6 independent experiments. Using mean Cp values from quantitative polymerase chain reaction, mRNA expression and DNA copy number were determined using the ∆Cp method. *MYCN, MYCN proto-oncogene bHLH transcription factor; B-myb, MYB proto-oncogene like 2; HPRT1, hypoxanthine phosphoribosyltransferase 1; E2F1, E2F transcription factor 1; hCdt1, chromatin licensing and DNA replication factor 1; GMNN, geminin; p27, cyclin-dependent kinase inhibitor 1B; CDK2, cyclin dependent kinase 2; p21, cyclin-dependent kinase inhibitor 1A; L3MBTL1, L3MBTL1 histone methyl-lysine binding protein; c-Myb, transcriptional activator Myb; A-myb, MYB proto-oncogene like 1; E2F2, E2F transcription factor 2.*
Correlations between 1p36 alterations and expression of c-myb, B-myb and p21 genes. Previous reports have revealed that c-Myb and B-Myb facilitate G2/M progression via the direct upregulation of cyclin B1 in normal, embryonic stem and cancer cells (47-49), whereas depletion of p21 causes chromosome segregation and cytokinesis defects in HCT116, HeLa and SAOS-2 cells (50). Statistical analyses of the present study revealed a significant negative correlation between p21 expression and 1p36 imbalance (r=−1.00, P<0.001; Fig. 4C); however, a significant positive correlation between c-myb expression and 1p36-3/3 alteration (r=0.96, P<0.02; Fig. 4D) in neuroblastoma cell lines was observed (Tables II and III). In addition, B-myb expression and 1p36-3/3 alteration were negatively correlated with 1p36 deletion (r=−0.82, P=0.089; r=−0.87, P=0.054; Tables II and III; Fig. 4E and F). All statistical data suggested that c-myb, B-myb and p21 genes may serve an important role against the genomic instability of chromosome 1p in neuroblastoma cells.

Using qPCR, MYCN gene copy number per haploid genome compared with the reference gene, p53, was notably high in Kelly cells (1,488.87 copies); however, 82.14, 80.45 and 74.54 copies were detected in IMR32, SIMA and MHH-NB-11 cells, respectively. The copy number for SH-SY5Y cells was 0.63 (Fig. 3C). Pearson’s correlation coefficient analysis revealed a notably positive significant correlation between 1p36-3/3 c-myb and E2F1 may be involved in controlling MYCN expression and amplification. The present study employed RNAi against c-myb mRNA. A c-myb-specific shRNA sequence was designed and ligated into a plasmid DNA vector. Following transformation, plasmid DNA was transfected into MYCN-amplified Kelly, IMR32, SIMA and MHH-NB-11 neuroblastoma cell lines. To generate stably transfected cell lines, the cells expressing c-myb shRNA were selected using G418. The presence of c-myb-specific and EGFP-specific shRNAs in plasmid vectors prior to transfection was confirmed by SpeI enzyme digestion and DNA sequencing.

RNAi reduced c-myb mRNA expression by 95.58, 77.79, 86.42 and 88.89% in Kelly, SIMA, MHH-NB-11 and IMR32 cells, respectively (Fig. 5A). MYCN/p53 DNA copy number ratios from MYCN-amplified cell lines were determined using the ΔΔCp method (36) as normalized to the Cp values of MYCN and p53 genes from the SH-SY5Y control cell line in pre- and post-c-myb RNAi groups. These results were compared with those of the efficiency corrected (dilution) method (36,38) in Kelly, SIMA and IMR32 cells. The results of the present study revealed that MYCN gene copy numbers increased by 115.85, 71.70, 85.31 and 53.69% following c-myb RNAi treatment of Kelly, SIMA, IMR32 and MHH-NB-11 cells, respectively (Fig. 5B).
Figure 4. Scatter plots for correlations of gene expressions and genomic alterations identified in neuroblastoma cell lines. The correlations of (A) MYCN copy number and 1p36-3/3 alteration, (B) c-myb and MYCN expression, (C) p21 expression and 1p36 imbalance, (D) 1p36-3/3 alteration and c-myb expression, (E) B-myb expression and 1p36 deletion, (F) 1p36 deletion and 1p36-3/3 alteration and (G) MYCN copy number and GMNN expression were presented. The scatter plots were produced using Excel. MYCN, MYCN proto-oncogene bHLH transcription factor; c-Myb, transcriptional activator Myb; B-myb, MYB proto-oncogene like 2; GMNN, geminin; p21, cyclin-dependent kinase inhibitor 1A.

Figure 5. The effect of c-myb RNAi treatment on MYCN gene copy number. (A) Interference rate of c-myb mRNA was presented as compared with shRNA controls in MYCN-amplified neuroblastoma cell lines. The expression levels of c-myb mRNA were determined pre- and post-c-myb RNAi treatment of Kelly, SIMA, MHH-NB-11 and IMR32 cell lines, as normalized to the Cp values of target and reference genes of the SH-SY5Y control cell line. R-value was calculated using the mean Cp ± standard error of duplicate or triplicate experiments in case of discordant results. (B) MYCN/p53 DNA copy number ratios were determined by the delta-delta Cp method pre- and post-c-myb RNAi treatment as compared with shRNA control in MYCN-amplified neuroblastoma cell lines. Data are expressed as mean Cp ± standard error of the mean from ≥6 independent experiments. *P<0.05, Wilcoxon signed rank test (one-tailed). shRNA, short hairpin RNA; c-myb, transcriptional activator myb; RNAi, RNA interference; MYCN, MYCN proto-oncogene bHLH transcription factor.
The expression levels of MYCN, c-myb, E2F1, hCdt1, B-myb, GMNN, p21 and L3MBTL1 genes in Kelly, SIMA, MHH-NB-11 and IMR32 cell lines as normalized to the Cp values of target and reference genes using the delta-delta Cp method (36). Data are expressed as mean variation (%) ± standard error of the mean from Kelly, SIMA, MHH-NB-11 and IMR32 cell lines (n=4). *P<0.05; Wilcoxon signed rank test (one-tailed). shRNA, short hairpin RNA; RNAi, RNA interference. c-myb cell lines.

Figure 6. Alterations in potential c-Myb target gene expression and MYCN gene copy number following treatment with c-myb RNAi. MYCN gene copy number and expression levels of c-myb and potential target genes (B-myb, p21, hCdt1, E2F1, L3MBTL1, GMNN and MYCN) were calculated in MYCN-amplified neuroblastoma cell lines as normalized to the Cp values of target and reference genes of SH-SY5Y control cell line using the delta-delta Cp method (36). "Data are expressed as mean variation (%) ± standard error of the mean from Kelly, SIMA, MHH-NB-11 and IMR32 cell lines (n=4). *P<0.05; Wilcoxon signed rank test (one-tailed). shRNA, short hairpin RNA; RNAi, RNA interference. c-myb amplified neuroblastoma cell lines (Fig. 6).

The expression levels of MYCN, c-myb, E2F1, hCdt1, B-myb, GMNN, p21 and L3MBTL1 genes in Kelly, SIMA, MHH-NB-11 and IMR32 cell lines as normalized to the Cp values of target and reference (HPRT1) genes of the control SH-SY5Y cell line were determined pre- and post-c-myb RNAi. The mRNA expression level of each gene was presented as the mean of related expression levels from the four aforementioned MYCN-amplified cell lines (Fig. 6). The expression level of MYCN was moderately decreased by 33.99±5.78% following treatment with c-myb RNAi in MYCN-amplified cell lines, while E2F1 expression was downregulated by 86.6±8.4%. These results suggested that c-myb may be involved in the induction of E2F1 and MYCN expression, potentially via putative c-Myb binding sites in their promoters. However, the c-myb may also be indirectly associated with MYCN expression via the upregulation of E2F1 (Fig. 7A and B). E2F1 may be involved in activation of the MYCN promoter, possibly via putative E2F1 binding sites (Fig. 1B). It was previously reported that E2F1-3 proteins activate the MYCN promoter via E2F binding sites in Kelly and IMR32 cells (51). Taken together, the findings of the present study indicate that c-myb and E2F1 may be involved in controlling MYCN expression and amplification in MYCN-amplified neuroblastoma cells.

E2F1 may contribute to hCdt1, p21 and B-myb gene expression in MYCN-amplified neuroblastoma cells. In HCT116 cells, the hCdt1 promoter is activated by E2F1 (52). In addition, E2F1 induces p21 transcription by directly binding to the proximal promoter of p21 via a p53-independent mechanism in NIH3T3 cells (53), but also transactivates the B-myb promoter in SAOS-2 cells (54).

In the present study, the mRNA expression level of hCdt1 declined by 37.48±12.45% following c-myb RNAi in MYCN-amplified neuroblastoma cell lines (Fig. 6). In addition, a significant positive correlation was identified.
between E2F1 and hCdt1 expression post-c-myb RNAi. p21 was also expressed; however, the p53 transcript was not detected in neuroblastoma cells (Table III). Following c-myb RNAi treatment, p21 expression was downregulated by 92.51±4.94% in MYCN-amplified neuroblastoma cell lines (Fig. 6). Additionally, B-myb expression was downregulated by 82.49±6.28% following c-myb RNAi treatment (Fig. 6).

These results suggest that E2F1 may induce hCdt1, p21 and B-myb expression, potentially via putative E2F1 binding sites in the promoters of these genes in MYCN-amplified neuroblastoma cells (Fig. 7).

**MYCN gene is amplified during S phase, possibly via a replication-based mechanism:** GMNN expression is upregulated following c-myb RNAi treatment in MYCN-amplified neuroblastoma cell lines. GMNN transcription is induced by E2F1-4 transcription factors via the RB transcriptional corepressor 1 (Rb)/E2F signaling pathway (52). The present study reported GMNN upregulation; however, E2F1 mRNA expression was downregulated following c-myb RNAi treatment (Fig. 6). GMNN was also upregulated following E2F1 RNAi treatment (data not shown) in MYCN-amplified neuroblastoma cell lines.

The mRNA expression levels of GMNN are higher in the S and G2/M phases compared with the G1/S transition of the cell cycle (28,52). In addition, Rb serves an important role in the repression of GMNN via intragenic E2F sites during G1 (55). The promoter of Rb contains a putative E2F1 binding site, suggesting that E2F1 may directly regulate the Rb promoter. Therefore, E2F1 may be associated with the repression of GMNN expression via the induction of Rb in MYCN-amplified neuroblastoma cells (Fig. 7B). These findings indicate that MYCN gene copy number increased during S phase, while neuroblastoma cells progress toward G2/M phase following c-myb RNAi treatment (Fig. 7B).

c-myb RNAi causes silencing of L3MBTL1 expression in MYCN-amplified cell lines, indicating G2/M arrest. Inhibition of L3MBTL1 mRNA expression leads to G2/M arrest in a variety of cancerous and normal human cells (25). In the present study, L3MBTL1 expression was silenced in MYCN-amplified cell lines following treatment with c-myb RNAi (Fig. 6). In addition, bioinformatics analysis demonstrated that the promoter of L3MBTL1 harbors a putative binding site for c-Myb, but not for E2F. These results suggest that c-myb may induce L3MBTL1 expression, possibly via a putative c-Myb binding site identified in the promoter of L3MBTL1 in MYCN-amplified neuroblastoma cells (Fig. 7B).

In addition, L3MBTL1 expression was highly down-regulated by E2F1 RNAi in Kelly, SIMA and IMR32 cells (data not shown), suggesting that E2F1 may transactivate the promoter of c-myb in neuroblastoma cells, similar to human glioblastoma cells (56). However, the c-myb promoter does not include any putative E2F binding sites.

In conclusion, the findings of the present study suggested that L3MBTL1 may be a potential c-Myb target gene. In addition, similar to GMNN upregulation, the silencing of L3MBTL1 expression following c-myb RNAi treatment indicates that MYCN gene copy number increased prior to G2/M arrest in MYCN-amplified neuroblastoma cell lines (Fig. 7B).

Taken together, it is concluded that MYCN gene is amplified during S phase, potentially via a replication-based mechanism.

**Discussion**

Neuroblastoma is a common pediatric solid tumor derived from the primitive cells of the sympathetic nervous system (57). MYCN amplification is an important factor associated with poor prognosis in neuroblastoma (5). MYCN is amplified in 18-38% of reported cases (3,5-7) and in a panel of neuroblastoma cell lines (8). The mRNA expression levels of MYCN and c-myb decline within 3 h following treatment with retinoic acid in neuroblastoma cell lines (16). However, the expression and amplification mechanisms of MYCN require further study.

The present study investigated the potential target genes of c-Myb and the effects of c-myb RNAi on MYCN expression and amplification. MYCN gene copy number and mean expression levels of MYCN, c-myb, E2F1, hCdt1, B-myb, GMNN, p21 and L3MBTL1 genes were determined in MYCN-amplified neuroblastoma cell lines, which were normalized to their counterparts from the SH-SY5Y control cell line in pre- and post-c-myb RNAi treatment. To compare with those of the ∆∆Cp method, MYCN gene copy numbers of Kelly, SIMA and IMR32 cells were also determined using the dilution method.

The ∆∆Cp method revealed that MYCN gene copy number increased by 115.85, 71.70, 85.31 and 53.69% in Kelly, SIMA, IMR32 and MHH-NB-11 cells, respectively, following c-myb RNAi treatment. The dilution method also revealed that MYCN gene copy number was increased in Kelly (336.49%), SIMA (20.58%) and IMR32 (76.83%) cells normalized to target and reference Cp values of the control SH-SY5Y cell line. However, PCR amplification efficiency (E) values out of range 1.95-2.05 in the dilution method led to notable differences in the MYCN gene copy numbers compared with those obtained from the ∆∆Cp method, particularly for Kelly and SIMA. Using the dilution method, E values >2.0, which are unexpected in theory, have been practically observed (36). Taken together, ∆∆Cp and dilution methods demonstrated an increase in MYCN gene copy number following c-myb RNAi treatment, suggesting that c-myb may be involved in controlling MYCN amplification in MYCN-amplified neuroblastoma cells.

Previously, Beall et al (21) reported that the protein product of D. melanogaster-myb gene, which is closely associated with the vertebrate myb gene family (including A-myb, B-myb and c-myb) (19,20), is required for chorion gene amplification in trans. However, it was proposed that a Dm Myb-MuvB repressor complex, including D-L(3)mbt protein, may be involved in the repression of transcription and DNA replication (23). The paralogous human gene, c-myb, has acquired novel functions not possessed by the Dm-myb gene (20). L3MBTL1, a human homolog of the D-l(3)mbt protein, interacts with the DNA sliding clamp and other proteins, forming a replicative helicase complex, and is required for progression of the replication fork (25). Loss of L3MBTL1 mRNA expression is an indicator of G2/M arrest (25). The present study reported that L3MBTL1 mRNA expression was silenced, whereas MYCN gene copy number increased following c-myb RNAi treatment of MYCN-amplified neuroblastoma cell lines. Taken together with published data,
the findings of the present study suggest that L3MBTL1 may act as a guard against arrest or abnormal forms of replication forks during MYCN amplification. Furthermore, the present study indicated that MYCN gene copy number was increased prior to G1/M arrest.

Statistical analysis revealed a notably significant positive correlation between c-myb and MYCN expression levels in neuroblastoma cells (Table III). Furthermore, the expression levels of MYCN were moderately decreased in MYCN-amplified neuroblastoma cells following c-myb RNAi treatment. In addition, bioinformatics analysis demonstrated that the enhancer and promoter of MYCN include putative c-Myb binding sites. The results of the present study suggest that MYCN may be a potential target gene of c-Myb and that c-myb may be associated with the induction of MYCN expression, potentially via the promoter and/or enhancer regions of MYCN. Upon induction by retinoic acid in human neuroblastoma cells, the mRNA expression levels of c-myb and MYCN decrease within 3 h (14-18).

Following treatment with c-myb RNAi, the mRNA expression levels of E2F1 and MYCN decreased, whereas the MYCN gene copy number increased in MYCN-amplified neuroblastoma cells. Bioinformatics analyses demonstrated that the MYCN promoter includes putative E2F1 binding sites, while that of E2F1 harbors c-Myb binding sites. These results suggested that E2F1 may be a potential target gene of c-Myb; and c-myb gene is involved in the induction of E2F1 expression potentially via a putative c-Myb-binding site in the E2F1 promoter. As with c-myb, E2F1 may also be associated with the induction of MYCN expression, potentially via E2F1 binding sites in the MYCN promoter. Our preliminary studies demonstrated that MYCN expression is downregulated by E2F1 RNAi in Kelly and IMR32 cells (data not shown). E2F1-3 have been reported to activate the proximal promoter of MYCN gene in Kelly and IMR32 cells (51). Collectively, these findings suggested that c-myb and E2F1 may be involved in controlling MYCN expression and amplification via the promoter and/or enhancer regions of MYCN.

In the present study, B-myb and p21 were observed to be downregulated following treatment with c-myb RNAi. In addition, the expression levels of B-myb and p21 decreased following E2F1 RNAi treatment in Kelly, SIMA, MHH-NB-11 and IMR32 cell lines (data not shown). Bioinformatics data revealed that the p21 promoter includes putative E2F1 binding sites without a c-Myb binding site (Qiagen, Inc.). These results indicated that E2F1 may be associated with the induction of B-myb and p21 mRNA expression, possibly via putative E2F1 binding sites in their respective promoters. A direct interaction causing S-phase arrest between the E2F1 transcription factor and the proximal promoter of p21 was previously demonstrated in NIH3T3 cells (53), and E2F1 transactivates the B-myb promoter in SAOS-2 cells (54).

E2F1 transcriptionally activates the Tp73 tumor suppressor that induces p53-responsive genes and apoptosis (58). The expression level of p21 protein is increased in the IGR-N-91 neuroblastoma cell line (containing mutated p53) infected with TAp73α recombinant adenovirus; this leads to G1 arrest via a p53-independent signaling pathway (59). In MYCN-amplified neuroblastoma, MYCN transactivates miR-17-5p, which in turn accelerates cell cycle progression and protects cells from apoptosis via the downregulation of p21 and BIM, respectively (60). MYCN overexpression sensitizes MYCN-amplified neuroblastoma cells to apoptosis via the induction of p53 (32). Additionally, enforced MYCN expression causes a significant reduction in cell viability via the apoptosis signaling pathway in SY5Y and SK-N-AS cell lines lacking MYCN amplification (61).

Furthermore, Guglielmi et al (18) reported that the mRNA expression levels of pro-apoptotic E2F1 and Tp73 are increased without modulating Tp53 mRNA expression, due to the inhibition of miR-9, miR-20a and miR-92a by upregulated MYCN expression in SK-N-AS cells. It was concluded that MYCN may be required during the activation of neuroblastoma differentiation to induce apoptosis in undifferentiated cells. In addition, Guglielmi et al (18) reported that cell death was not observed following treatment with retinoic acid of MYCN-silenced LAN-5 cells (~40%) harboring MYCN amplification, whereas control LAN-5 cells did exhibit retinoic acid-induced apoptosis.

In the present study, p21 was identified to be transcribed in neuroblastoma cells, whereas p53 mRNA was not expressed under normal conditions. Taken together, the results suggested that c-myb and E2F1 may contribute to the sensitization of MYCN-amplified cells to apoptosis by inducing MYCN overexpression that indirectly activates E2F1 protein, which in turn upregulates p73 and p21 in neuroblastoma (18,32,53,58,62-64). That is, MYCN amplification may be partially controlled via E2F1-induced G1/S arrest and apoptosis in MYCN-amplified neuroblastoma cells (Fig. 7A).

In a previous study, c-Myc expression was reported to be upregulated in 5-15% of the p53−/− mice cells in all the tissues examined, whereas <1% of p53+/− cells express detectable levels of c-Myc protein (65). Apoptotic p53−/− cells characterizedly exhibit atypical chromosome morphology, abnormally amplified centrosomes, aneuploidy, c-myc gene amplification and elevated c-Myc protein levels, indicating that c-Myc-overexpressing cells undergo apoptosis and numerous genetically aberrant cells are eliminated by p53-independent apoptosis in vivo (65).

The apopotic control mechanism proposed in the present study for MYCN-amplified neuroblastoma cells via c-myb and E2F1-associated MYCN overexpression can explain why the MYCN gene copy number increases despite reductions in mRNA expression levels of MYCN following c-myb RNAi treatment. In cisplatin-resistant UFK-NB-4 neuroblastoma cells with MYCN amplification, MYCN expression levels increased although no significant change was observed in the MYCN copy number following cisplatin treatment, suggesting that alterations in MYCN expression may not always be associated with variations in MYCN copy number (66). MYCN expression may be controlled by mechanisms independent from MYCN copy number under discrete conditions in neuroblastoma cell lines. Additional MYCN gene copies may also repress their own transcription. By analyzing genome-wide data of the regions that vary in copy number in humans and certain model organisms, it has been reported that genes with varied copy number in copy number variation (CNV) regions are expressed at lower and more variable levels than genes mapped elsewhere; CNVs also exert a global influence on
the transcriptome (67). Alterations in copy number of CNV regions can influence gene expression by several mechanisms, including physical dissociation of the transcription unit from its cis-acting regulators, modification of transcriptional control by altering chromatin structure and position, and perturbation of transcript structure (67). Additional copies of MYCN may also impair MYCN transcription due to steric hindrance for access to specific transcription factories, as previously described in a general hypothesis regarding extra copies of any gene (67,68).

In the present study, FISH analyses of the metaphase spreads from neuroblastoma cells revealed near-diploid (2n±), hyperhaploid (n+) and near-polyplid (3n±, 3n+ and 4n-) aneuploidies. In addition, qPCR experiments revealed the expression of CDK2 and MYCN genes in neuroblastoma cells. Centrosomes with multiple copies, abnormal structure and function have frequently been observed in the most common types of human malignant tumors and tumor-derived cell lines (69). Hyperactive CDK2 and MYCN overexpression induce centrosome amplification and chromosomal instability, resulting in aneuploidy and formation of micronuclei (a precursor to aneuploidy) in p53− mouse embryonic fibroblasts and neuroblastoma cells following DNA damage, respectively (70,71). However, enhanced expression of MYCN without DNA damage in a neuroblastoma cell line did not cause centrosome hyperamplification and micronuclei formation (71).

Sugihara et al (71) predicted an increase in a fraction of the cell population with DNA contents of 8N, resulting from centrosome hyperamplification with DNA replication caused by the failure of cell division following DNA damage. However, Sugihara et al (71) also identified that the fraction of MYCN-EGFP cells with 8N did not increase compared with that of vector-EGFP cells, suggesting that MYCN overexpression may contribute to the apoptotic elimination of neuroblastoma cells with genomic DNA amplification and formation of micronuclei. Similarly, the apoptotic cells of p53− mice contain abnormally amplified centrosomes, aneuploidy, high levels of c-Myc expression and gene amplification (65).

Statistical analyses revealed that B-myb and p21 expression are negatively correlated with 1p36 deletion and imbalance, respectively (r=−0.82, P=0.089; r=−1.00, P<0.001). Conversely, a significant positive correlation was identified between c-myb expression and 1p36/3/3 alteration in neuroblastoma cells (r=0.96, P<0.02), suggesting that c-myb, B-myb and p21 may serve a role against genomic instability in chromosome 1p. p21 deficiency leads to abnormal centriole replication (72), whereas p21 overexpression rescues p53-deficient human tumor cells from endoreduplication and aneuploidy/polyplid (73,74).

Following c-myb RNAi treatment, L3MBTL1 expression was silenced in MYCN-amplified Kelly, SIMA, IMR32 and MHH-NB-11 neuroblastoma cell lines in the current study, indicating that L3MBTL1 may be a potential c-Myb target gene. The c-myb gene may be involved in the induction of L3MBTL1 expression, possibly via a putative c-Myb-binding site in the L3MBTL1 promoter. In certain cancer cell lines, including SW480, the L3MBTL1 transcript is markedly reduced (24). In addition, L3MBTL1 has been considered to be a candidate tumor suppressor gene in myeloid malignancies associated with 20q12 deletions (25). Furthermore, depletion of L3MBTL1 causes replicative stress, DNA breaks, activation of the DNA damage response and genomic instability in human cells, and leads to G1/M arrest in a variety of cancerous and normal human cells (25). Considering the published literature, the results of the present study suggested that L3MBTL1 may act as a tumor suppressor gene in neuroblastoma cells. Together, the results indicated that MYCN gene copy number increased prior to G1/M arrest, potentially following the loss of L3MBTL1 mRNA expression on treatment with c-myb RNAi.

GMNN expression was also increased along with MYCN gene copy number following c-myb RNAi treatment in MYCN-amplified neuroblastoma cell lines in the current study. In addition, a positive correlation was identified between GMNN expression and MYCN gene copy number. These results suggest that GMNN upregulation may lead to a switch from genomic DNA replication to MYCN amplification by suppressing re-replication. Similarly, in the follicle cells of Dm geminin mutant ovaries, it was reported that a switch from general genomic endoreplication to the amplification cycles occurs during stage 10B (75). The present study also reported a partially identical region of −44% between DNA fragments encompassing the sequence upstream of human MYCN gene and ACE3 controlling chorion gene amplification in D. melanogaster. This finding suggested that the expression and/or amplification mechanisms of developmentally-regulated genes may be conserved among different organisms during the evolutionary process. As with c-myb RNAi, E2F1 RNAi caused the upregulation of GMNN expression in MYCN-amplified neuroblastoma cell lines (data not shown). GMNN expression is transcriptionally repressed by the activation of Rb throughout G1 (55). In view of the literature and the findings of the present study, it can be hypothesized that the E2F1 gene may be indirectly involved in controlling GMNN expression via transcriptional activation of the Rb promoter.

In addition, the results of the present study suggested that E2F1 contributed to the induction of hCdt1 expression in MYCN-amplified neuroblastoma cells. Additionally, it was identified that an E2F1 RNAi experiment in SIMA, as with c-myb RNAi, caused the downregulation of hCdt1 expression (data not shown). The promoter of hCdt1 is activated by E2F1 in HCT116 cells (52). Taken together, the results suggested that E2F1 may be implicated in controlling the expression of GMNN and hCdt1 genes throughout the cell cycle (Fig. 7B).

The mRNA expression levels of GMNN are upregulated in S and G2/M phases (28,52), followed by the degradation of geminin protein via APC/CICIC and mitotic spindle checkpoint during metaphase and early anaphase (26,76) in human cells. In addition, loss of L3MBTL1 transcript leads to G1/M arrest (25). B-Myb and c-Myb contribute to the G1/M transition in normal human cells, embryonic stem cells and cancer cells (47-49).

In conclusion, the results of the present study revealed that c-myb and E2F1 genes may be involved in controlling MYCN expression and amplification in MYCN-amplified neuroblastoma cells. As described in the model mechanism proposed in Fig. 7A, c-myb and E2F1 may contribute to the apoptotic elimination of MYCN-amplified cells via the induction of MYCN overexpression that indirectly activates E2F1-induced apoptosis. However, it remains to be determined whether c-myb and E2F1 are involved in the induction
of apoptosis in MYCN-amplified neuroblastoma cell lines. In addition, c-myb may be associated with the upregulation of L3MBTL1 (Fig. 7B). Furthermore, L3MBTL1 may be considered as candidate tumor suppressor gene in neuroblastoma cells. The results of the present study also indicated that the cell cycle is stopped or delayed in the G2/M transition of the MYCN-amplified neuroblastoma cells following c-myb RNAi treatment (Fig. 7B). Therefore, it is concluded that MYCN gene is amplified during S phase, possibly via a replication-based mechanism (Fig. 7B).

In drug-resistant Chinese hamster sublines and two neuroblastoma cell lines, SK-N-BE(2) and IMR-32, the HSRs containing DHFR and MYCN, respectively, were previously demonstrated to undergo relatively rapid and synchronous replications prior to the midpoint of the S phase as observed by tritiated thymidine radioautography (77). However, there is no clear evidence on whether MYCN is amplified during S phase. Neuroblastoma cell lines harbor a head-to-tail tandem array in the direct orientation of DNA segments containing MYCN gene in amplified regions (78), suggesting that MYCN amplification may arise from a mechanism other than those involving breakage-fusion-bridge cycles that produce inverted duplications (79). Numerous replication-based mechanisms for the formation of MYCN or general gene amplification have been proposed (12,41,78,80-84).

Aygun (84) recently reported that long inverted repeats (LIRs) are significantly associated with 5' and 3' boundary regions of the amplicon units containing MYCN gene in 14 neuroblastoma cell lines and 42 other primary solid tumors, whose genomic data were obtained from the catalogue of somatic mutations in cancer (COSMIC database; cancer.sanger.ac.uk/cosmic/) (85). In addition, Aygun (86) previously reported a significant association between LIRs and the breakpoint regions of gross deletions in human cancers and inherited diseases. Numerous LIRs inside and outside the MYCN amplicon units were identified, suggesting that LIRs may extrude hairpin and cruciform structures in single- and double-stranded DNA during replication, respectively (84). The hairpin structure can form at an interrupted LIR on leading and lagging strand templates simultaneously during replication (87). The hairpin and cruciform conformations can cause replication fork stalling (87,88). In addition, a mean microhomology of 5.18 bp (range, 2-14 bp) between DNA sequences of 150 bp encompassing the 5' and 3' boundaries of the MYCN amplicon units has been reported (12,84). Microhomology between 0 and 15 bp indicates nonhomologous end joining, microhomology-mediated end joining, microhomology-mediated break-induced replication or fork stalling and template switching mechanisms (83,89,90). Consequently, the findings of the present study indicated that a replication-based mechanism involving LIRs in a microhomology-dependent manner may generate MYCN amplification during S phase. Therapeutic targeting of MYCN amplification during S phase may improve the prognosis of neuroblastoma patients.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NA conceived the study, performed the experiments, analyzed the data, wrote the manuscript and prepared all figures and tables. OA supervised the project and conducted the research.

Ethics approval and consent to participate

All the experiments were performed in established human neuroblastoma cell lines. The present study was approved by the local Ethics Committee of Clinical and Laboratory Research of the Faculty of Medicine, Dokuz Eylul University (04.10.2005/223, protocol no. 188).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY and Hammond D: Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med 313: 1111-1116, 1985.
2. Maris JM and Matthay KK: Molecular biology of neuroblastoma. J Clin Oncol 17: 2264-2279, 1999.
3. Altungoz O, Aygun N, Tumer S, Ozer E, Olgun N and Sakizli M: Correlation of modified Shimada classification with MYCN and lp36 status detected by fluorescent in situ hybridization in neuroblastoma. Cancer Genet Cytogenet 172: 113-119, 2007.
4. Gurney JG, Ross JA, Wall DA, Bleyer WA, Severson RK and Robison LL: Infant cancer in the U.S.: Histology-specific incidence and trends, 1973 to 1992. J Pediatr Hematol Oncol 19: 428-432, 1997.
5. Brodeur GM, Seeger RC, Schwab M, Varmus HE and Bishop JM: Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science 224: 1121-1124, 1984.
6. Bartram CR and Berthold F: Amplification and expression of the N-myc gene in neuroblastoma. Eur J Pediatr 146: 162-165, 1987.
7. Valent A, Guillaud-Bataille M, Farra C, Lozach F, Spengler B, Terrier-Lacombe MJ, Valteau-Couanet D, Danglot G, Lenoir GM, Brison O and Bernheim A: Alternative pathways of MYCN gene copy number increase in primary neuroblastoma tumors. Cancer Genet Cytogenet 153: 10-15, 2004.
8. Schwab M, Alitalo K, Klemplnauer KH, Varmus HE, Bishop JM, Gilbert F, Brodeur G, Goldstein M and Trent J: Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 305: 245-248, 1983.
9. Edsjö A, Nilsson H, Vandesompele J, Karlsson J, Pattyn F, Culp LA, Spleeman F and Pålhamn S: Neuroblastoma cells with overexpressed MYCN retain their capacity to undergo neuronal differentiation. Lab Invest 84: 406-417, 2004.

10. White PS, Thompson PM, Gotot T, Okawa ER, Igarashi J, Kok M, Winter C, Gregory SG, Hogarty MD, Maris JM and Brodeur GM: Definition and characterization of a region of 1p36.3 consistently deleted in neuroblastoma. Oncogene 24: 2644-2651, 2005.

11. Henrich KO, Schwab M and Westermann F: 1p36 tumor suppression-a matter of dosage? Cancer Res 72: 6079-6088, 2012.

12. Aygun N: Biological and genetic features of neuroblastoma and their clinical importance. Curr Pediatr Rev 14: 73-90, 2018.

13. Vattioni M, Migliorini A, Skorski T, Pucci S, Nieborowska-Skorska M, Romeo A and Calabretta B: Inhibition of proliferation by c-myb antisense RNA and oligodeoxynucleotides in transformed neuroectodermal cell lines. Cancer Res 52: 4221-4226, 1992.

14. Thiele CJ, Reynolds CP and Israel MA: Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. Nature 313: 404-406, 1985.

15. Thiele CJ, Cohen PS and Israel MA: Regulation of c-myb expression in human neuroblastoma cells during retinoic acid-induced differentiation. Mol Cell Biol 8: 1677-1683, 1988.

16. Abemayer E and Sidell N: Human neuroblastoma cell lines as models for the in vitro study of neoplastic and neuronal cell differentiation. Environ Health Perspect 80: 3-15, 1989.

17. Aktas S, Altun Z, Erbayraktar Z, Aygun N and Olgun N: Effect of cytosine arabinoside and retinoic acid on MYC-N protein expression in neuroblastoma. Appl Immunohistochem Mol Morphol 18: 86-89, 2010.

18. Gagliardi L, Cinnella C, Nardella M, Maresca G, Valenti A, Mercanti D, Felsani A and DaGnanno I: MYCN gene expression is required for the onset of the differentiation programme in neuroblastoma cells. Cell Death Dis 5: e1081, 2014.

19. Nomura N, Takahashi M, Matsui M, Ishii S, Date T, Sasamoto S and Koga H, Matsui S, Hirota T, Takebayashi S, Okumura K and Botchan MR: Identification of a sequence-specific replicator and origin elements are required for site-specific DNA replication. Nature 420: 833-837, 2002.

20. Fujita M: Myb in neuroblastoma. Proc Natl Acad Sci USA 107: 22552-22557, 2010.

21. Liu DX, Biswas SC and Greene LA: B-Myb and C-Myb play required roles in neuronal apoptosis evoked by nerve growth factor deprivation and DNA damage. J Neurosci 24: 8720-8725, 2004.

22. Aygun N and Altungoz O: Novel structural abnormalities involving chromosomes 1, 7 and 2 identified by fluorescence in situ hybridization (FISH) and/or cytogenetic karyotyping in Kelly and SH-SY5Y human neuroblastoma cell lines, respectively. J Can Res Updates 6: 46-55, 2017.

23. Protocol for DNA extraction. DNA preparation from adherent cells. Section of Cancer Genomics, Genetics Branch, NCI National Institutes of Health. Available from: https://ccr.cancer.gov/sites/default/files/dna_prep_from_adherent_cells.pdf.

24. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45, 2001.

25. Botchan MR: Identification of a genes, A-myb and B-myb. Nucleic Acids Res 16: 11075-11089, 1988.

26. Lewis PW, Beall EL, Fleischer TC, Georlette D, Link AJ and Lewis PS, Thompson PM, Gotoh T, Okawa ER, Igarashi J, Hiller S, Breit S, Wang ZQ, Wagner EF and Schwab M: Localization of regulatory elements controlling human MYCN expression. Oncogene 6: 969-977, 1991.

27. Tower J: Developmental gene amplification and origin regulation. Annu Rev Genet 35: 273-304, 2001.

28. Wright JA, Smith HS, Watt FM, Hancock MC, Hudson DL and Stark GR: DNA amplification is rare in normal human cells. Proc Natl Acad Sci USA 87: 1791-1795, 1990.

29. Tanimura T, Ishi H, Goto Y, Watanabe S, Hara I, Kakinuma H, Tsuruoka Y, Oka Y and Sugimura T: Identification of a novel structural abnormality in a biopsy sample of a Japanese patient with acute myelogenous leukemia. Genes Chromosomes Cancer 37: 76-84, 2003.

30. Gavrieli Y, Dinstein S, Stanciu C, Szafranski H, Celniker Z, Ben-David D, Albright J, Lichtenstein A, Shuster D, Gossen J, Etkin A, Weinfeld M, Sitt J, Rubin E, Helling CR, Stetler-Stevenson W, Schachter D, Spiro R, Greenberg A, Weksberg R, Hecht H, Marmorstein R, Goodrich J, Where et al: c-myc protein expression is down-regulated upon cell cycle exit and are over-expressed in cancer-derived cell lines. Eur J Biochem 271: 3368-3378, 2004.

31. Aygun N and Altungoz O: c-myb AND MYCN IN MYCN-AMPLIFIED NEUROBLASTOMA CELLS.
relaxed microtubule damage checkpoints in human hematopoietic nuclear architecture, centriole overduplication, polyploidy, and hyperamplification after DNA damage in neuroblastoma cells. Miwa M: Enhanced expression of MYCN leads to centrosome cycle and are critical mediators of centrosome amplification instability in malignant tumors. Cancer Res 58: 3974-3985, 1998. Quesenberry P and Doxsey SJ: Centrosome defects and genetic transcription factories in large-scale structure and dynamics of transcription by forming hairpin structures on both leading and lagging strands. Genes Cells 21: 136-145, 2016. Amler LC and Schwab M: Amplified N-myc in human neuroblastoma cells is often arranged as clustered tandem repeats of differently recombined DNA. Mol Cell Biol 9: 4903-4913, 1989. Lo AW, Sabatier L, Fouldai B, Pottier G, Ricoul M and Murnane JP: DNA amplification by breakage/fusion/bridge cycles initiated by spontaneous telomere loss in a human cancer cell line. Neoplasia 4: 531-538, 2002. Schwarz M: Human neuroblastoma: From basic science to clinical debut of cellular oncosenes. Naturwissenschaften 86: 71-78, 1999. Watanabe T, Tanabe H and Horiiuchi T: Gene amplification system based on double rolling-circle replication as a model for oncogene-type amplification. Nucleic Acids Res 39: e106, 2011. Blumrich A, Zapatka M, Brueckner LM, Zheng D, Schwab M and Saveleya L: The FRA2C common fragile site maps to the borders of MYCN amplicons in neuroblastoma and is associated with gross chromosomal rearrangements in different cancers. Hum Mol Genet 20: 1488-1501, 2011. Slack A, Thornton PC, Magner DB, Rosenberg SM and Hastings PF: On the mechanism of gene amplification induced under stress in Escherichia coli. PLoS Genet 2: e48, 2006. Ayyun N: Acquired chromosomal abnormalities and their potential formation mechanisms in solid tumours. In: Chromosomal abnormalities—a hallmark manifestation of genomic instability. Larramendy M, (ed.) IntTech, pp27-57, 2017. Available from: https://www.intechopen.com/books/chromosomal-abnormalities-a-hallmark-manifestation-of-genomic-instabilityacquired-chromosomal-abnormalities-and-their-potential-formation-mechanisms-in-solid-tumours. Forbes SA, Beare D, Boutselas H, Bamford S, Bindal N, Tate J, Cole CG, Ward S, Dawson E, Ponting L, et al: COSMIC: Somatic cancer genetics at high-resolution. Nucleic Acids Res 45: D777-D783, 2017. Ayyun N: Correlations between long inverted repeat (LIR) features, deletion size and distance from breakpoint in human gross gene deletions. Sci Rep 5: 8300, 2015. Lai PJ, Lim CT, Le HP, Katayama T, Leach DR, Furukohri A and Maki H: Long inverted repeat transiently stalls DNA replication by forming hairpin structures on both leading and lagging strands. Genes Cells 21: 136-145, 2016. Voinneagu I, Narayanan V, Lobachev KS and Mirkin SM: Replication stalling at unstable inverted repeats: Interplay between DNA hairpins and fork stabilizing proteins. Proc Natl Acad Sci USA 105: 9936-9941, 2008. Conrad DF, Bird C, Blackburne B, Lindsay S, Mamanova L, Lee C, Turner DJ and Hurles ME: Mutation spectrum revealed by breakpoint sequencing of human germline CNVs. Nat Genet 42: 385-391, 2010. Hastings PJ, Ira G and Lupski JR: A microhomology-mediated break-induced replication model for the origin of human copy number variation. PLoS Genet 5: e1000327, 2009.