EUROPEAN JOURNAL OF CANCER 43 (2007) 2413–2422

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Direct regulation of the minichromosome maintenance complex by MYCN in neuroblastoma

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ARTICLE INFO

Article history:
Received 21 May 2007
Accepted 18 July 2007
Available online 10 September 2007

Keywords:
DNA replication
MCM
Neuroblastoma
MYCN

ABSTRACT

The c-Myc and MYCN oncogenes strongly induce cell proliferation. Although a limited series of cell cycle genes were found to be induced by the myc transcription factors, it is still unclear how they mediate the proliferative phenotype. We therefore analysed a neuroblastoma cell line with inducible *MYCN* expression. We found that all members of the minichromosome maintenance complex (MCM2–7) and MCM8 and MCM10 were up-regulated by MYCN. Expression profiling of 110 neuroblastoma tumours revealed that these genes strongly correlated with *MYCN* expression *in vivo*. Extensive chromatin immunoprecipitation experiments were performed to investigate whether the MCM genes were primary MYCN targets. MYCN was bound to the proximal promoters of the MCM2 to -8 genes. These data suggest that MYCN stimulates the expression of not only MCM7, which is a well defined MYCN target gene, but also of the complete minichromosome maintenance complex.

1. Introduction

Members of the Myc oncogene family are activated in many tumour types and can induce strong changes in the cellular phenotype. They stimulate cell growth, proliferation and invasiveness upon ectopic expression. The Myc oncogene family members, MYC, MYCN and MYCL, are basic-helix–loop–helix-leucine zipper domain containing transcription factors, which activate, together with their dimerization partner Max, gene expression of a number of genes in an E-box (CAC(A/G)TG) dependent manner.[1,2] MYCN and c-myc probably have very similar molecular functions. Mice in which the coding sequence of *MYC* was replaced by that of *MYCN* developed normally. This indicates that MYCN and c-myc can functionally replace each other and control the same cellular processes.[3]

High throughput mRNA profiling studies have identified many genes which are induced upon myc activation, but direct regulation by the myc transcription factors was established for only a limited number of genes. These myc-induced changes in gene expression start to explain the phenotypic changes known to result from myc activation. The Drosophila *MYC* gene promotes cell growth and indeed mammalian c-myc stimulates expression of several genes involved in protein synthesis.[4,5] We observed that MYCN and c-myc stimulate expression of proteins involved in ribosomal RNA processing and ribosome biogenesis.[6] The role of myc oncogenes in cell adhesion and tumour invasion[7–9] is in line with our recent observation that MYCN down-regulates the expression of many genes involved in cell–matrix interactions and in cytoskeleton architecture.[10] Most research has focused on the effect of myc oncogenes on cell proliferation. Indeed a

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doi:10.1016/j.ejca.2007.07.024

number of c-myc target genes are regulators of the cell cycle. c-Myc directly induces the expression of *cyclin-D2* and *CDK4*, eventually leading to increased cyclin-E-CDK2 activity and G1- to S-phase transition.[11,12] Also other cyclins are regulated by c-myc, including *Cyclin-A2*, *-B1*, *-D3* and *-E*.[13–17] In addition, a number of cell cycle genes are transcriptionally suppressed by myc. The cell-cycle inhibitory genes $p15^{INK4B}$ and $p21^{CIP1}$ are repressed by c-myc through interaction with the transcription factor Miz-1 at the Inr sequence in the core promoter.[18,19]

The *MYCN* gene is amplified in 20% of neuroblastoma tumours.[20] Neuroblastoma is a malignant childhood tumour of the sympathetic nervous system with a highly variable prognosis. Amplification of *MYCN* confers a poor prognosis[21] and ectopic expression of *MYCN* in neuroblastoma cell lines was found to stimulate cell cycle progression.[22,23] In this study, we generated gene expression profiles of a neuroblastoma cell line in which ectopic *MYCN* expression can be regulated. Strikingly, all minichromosome maintenance (MCM) genes were induced by MYCN. Chromatin immunoprecipitation (ChIP) experiments identified MCM2 to -8 as direct targets of MYCN. Moreover, microarray analyses of neuroblastoma tumours showed a strong correlation between expression levels of *MYCN* and all MCM genes. These results suggest that MYCN might stimulate the initiation of DNA replication and the accompanying G_1- to S-phase transition by induction of the whole minichromosome maintenance complex.

2. Materials and methods

2.1. Cell lines

The SHEP-21N cell line was grown in RPMI 1640 medium (GIBCO), supplemented with 10% foetal calf serum, 4 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin.[22] MYCN expression was switched off by the addition of 50 ng/ml tetracycline. For serum starvation, SHEP-21N cells were incubated for 36 h with the above indicated medium, but without foetal calf serum. Cells were serum stimulated by the addition of fetal calf serum to a final concentration of 10%.

2.2. Oligonucleotide microarray hybridization and analysis

Total RNA of cell lines was isolated by the LiCl-ureum method.[24] Total RNA of neuroblastoma tumours was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol.

RNA concentration and quality were determined using the ND-1000 spectrophotometer (NanoDrop, Wilmington, USA). RNA purification was performed using the RNeasy mini kit (Qiagen, Germantown, USA). Fragmentation of cRNA, hybridization to HG-U133 Plus 2.0 microarrays and scanning were carried out according to the manufacturer's protocol (Affymetrix Inc. Santa Barbara, USA) at the Microarray Department of the Swammerdam Institute of Life Science of the University of Amsterdam. Intensity values and the corresponding detection *p*-values were assigned to each probe set using the MAS5.0 algorithm of GCOS software (Affymetrix Inc. Santa Barbara, USA). The ratios of expression between two time points and their corresponding *p*-values were calculated present-call was <0.01 and the *p*-value of the ratio between the expression at $t = 0$ h and $t = 8$ h was <0.0005. Furthermore, the fold induction had to be >2 or <−2. If more than one probe-set belonged to the same gene, we assigned the expression data of the probe-set with the best *p*-value of the ratio between the two time points to that particular gene. Additional analyses of the microarray data were performed using the RMA and GCRMA normalisation algorithms. With RMA we found 105 of the 109 cell cycle genes described in this report, while with GCRMA we found 108 genes.

2.3. Northern blot analysis

Twenty micrograms of RNA was separated on a 0.8% agarose gel with 6.7% formaldehyde and blotted on Hybond N membranes in the presence of 10× SCC. Sequence-verified DNA probes were hybridized to filters overnight in 0.5 M $NaHPO_4$, pH 7.0, 7% SDS and 1 mM EDTA (incubation buffer) at 65 °C. *MCM* probes were hybridized in the presence of 100 μg human placenta DNA. Filters were washed two times in 40 mM $NaHPO_4$, 1% SDS and one time in 40 mM $NaHPO_4$ at 65 °C. Primers used for probe PCR are listed in the supplementary data.

2.4. Quantitative PCR

For first-strand cDNA synthesis 1 μg of total RNA was reverse transcribed using 125 pM oligodT_{12} primers, 0.5 mM dNTPs, 2 mM $MgCl_2$, RT-buffer (Invitrogen) and 100 U superscript II (Invitrogen) in a total volume of 25 μl.[25] cDNA was diluted two times to a total volume of 50 μl. A fluorescence-based kinetic real-time PCR was performed using the real-time iCycler PCR platform (Biorad, Hercules, USA) in combination with the intercalating fluorescent dye SYBR Green I. Twenty nanograms of cDNA was used for each quantitative PCR reaction. The IQ SYBR Green I Supermix (BioRad, Hercules, USA) was used in accordance with the manufacturer's instructions. A complete list of all the primers used for quantitative PCR is listed in the supplementary data. Expression values were normalised according to Vandesompele et al.[26] by geometric averaging of the genes β-actin and *PSMB4*. The expression of both β-actin and *PSMB4* genes was not regulated in the SHEP-21N cell line according to the microarray data.

2.5. Chromatin immunoprecipitation

SHEP-21N cells were grown in RPMI 1640 supplemented with 50 ng/ml tetracycline for two weeks. Tetracycline was removed by washing the cells three times with HANKS' balanced salts solution (HBSS) and cells were grown on RPMI 1640 for three more days. Cells (3×10^7) were used for four ChIPs (including two negative ChIPs with control antibody). We used a modified protocol of the Upstate ChIP kit (Upstate, Charlottesville, USA). The most important modification was that, after cross-linking and prior to the initial lysis step, nuclei were collected. Nuclei were obtained by harvesting cells in 1 ml Swelling Buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet-P40), incubation on ice for 30 min, homogenation using a syringe and needle (23G) and centrifugation