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DIFFERENCES IN GENE EXPRESSION AND MOLECULAR PATHWAY REGULATION BETWEEN MYCN AMPLIFIED AND 2P GAIN NEUROBLASTOMA TUMORS

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Abstract – *Objective:* *The malignancy of neuroblastoma (NB) is strongly connected with* MYCN *oncogene status. The gene expression profile was investigated in three subtypes of NB related to* MYCN *status (amplification - MNA, 2p gain and normal) in order to identify new candidate genes and to elucidate development of more aggressive forms of this pediatric tumor.*

Materials and Methods: *Human whole genome oligonucleotide expression microarrays were applied in the study.*

Results: *Hierarchical clustering analysis presented two distinct gene expression patterns corresponding to cases with and without MNA. For the first time, the 7 most upregulated genes and the 13 most downregulated genes in the MNA subgroup were selected in comparison to 2p gain tumors.*

Conclusions: *The obtained result demonstrates that* MYCN *has a significant impact on genome-wide NB gene expression. Increasing* MYCN *level promotes cell growth and motility while counteracting differentiation and attachment. Interestingly, NB with 2p gain, in comparison to MNA and normal* MYCN *status, showed a higher expression level of genes involved in cell migration but downregulated genes involved in nervous system development. This finding may indicate that 2p gain tumors have more aggressive behavior with a higher tendency toward metastasis than MNA cases.*

KEYWORDS: *2p gain, Gene expression, Molecular pathway, MYCN amplification, Neuroblastoma.*

INTRODUCTION

Cancer can be described as a disease of altered gene expression. Molecular stratification of tumors by gene expression profiling was applied to a large number of human malignancies as a tool for developing prognostic factors and personalized treatment[1,2]. In the present study, a microarray gene expression profile was used to explore the relationship between *MYCN* oncogene status and neuroblastoma biology and to provide a preliminary theoretical basis to search for biomarkers of malignant progression and new molecular therapeutic pathways.

Neuroblastoma (NB) is a solid tumor typically occurring during childhood. The detection frequency peak of NB is below 5 years of age[3-5]. NB is the most common extracranial malignant solid tumor arising from progenitor neural crest cells. Primary neoplastic lesions are located in the: abdomen (60-80%), chest (15%), neck (2-5%), pelvis (2-5%) with a tendency to tendency toward distant metastasis[3-5]. The malignancy of NB is very strongly connected with *MYCN* oncogene status. A poor outcome in NB is associated with *MYCN* amplification (MNA), whereas patients possessing a single copy of *MYCN* usually have a favorable prognosis. Approximately 25% of all NB cases are

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affected with MNA. This chromosomal alteration is the most significant unfavorable genetic factor correlated with high progression risk[5-10]. The Myc family containing *MYCN* is a group of transcription factors that play a critical role in regulating metastasis molecular pathways concerned with cell adhesion, motility, invasion, and degradation of extracellular matrix[10,11]. Therefore, the statement that *MYCN* has a profound effect on NB cell behavior is indisputable. Besides MNA, also "low-level" *MYCN* variants, like the gain of the *MYCN locus* on the short arm of chromosome 2 (2p24) named "2p gain", have been detected in NB[12-15]. Knowledge about associations regarding 2p gain and NB patient outcome is still insufficient, and its clinical significance is unclear. Therefore, the gene expression profile in three subtypes of NB were examined in order to identify new candidate genes, that may be related to *MYCN* status and the development of more aggressive forms of this pediatric tumor. Microarray gene expression profiling was used as an efficient and effective tool for the classification of NB on the basis of transcriptional patterns.

MATERIALS AND METHODS

Tumor tissue samples

In this study, 15 NB tumor tissue samples were collected from patients diagnosed in the Department of Pediatric Oncology and Hematology at the University Children's Hospital in Krakow from 2011 to 2016. All patients who had not been treated with radiotherapy or chemotherapy prior to surgery were included. Children with NB were enrolled to the study based on the result of a fluorescence *in situ* hybridization test for *MYCN* status. Patients were divided into 3 subtypes, according to international guidelines[16]: with MNA (n = 5) (Figure 1a), with 2p gain (n = 5) (Figure 1b), with normal *MYCN* status (n = 5) (Figure 1c). The clinical disease stage assessed according to the International Neuroblastoma Staging System (INSS), risk group and age of the study cohort for expression analysis were included in Table 1. The study was approved by the Ethics Committee.

Tissue sample collection

NB tumor tissue samples (0.2-0.5 cm^3) obtained after surgeries were immediately washed with 0.9% sodium chloride (NaCl) RNase-free saline and stored at −80°C for further testing.

RNA sample preparation

Total RNA was extracted from NB samples using TRIzol (Invitrogen, Thermo Fisher Scientific Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and quality of total RNA were measured by ultraviolet absorbance (NanoDrop® ND-1000 UV-Vis Spectrophotometer, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Screening gene expression profiles

Human whole genome oligonucleotide microarrays were applied in the study. Sample labeling and hybridization were performed in accordance with the SurePrint G3 Human Gene Expression 8x60K v2 Microarray Kit (Agilent Technologies, Santa Clara, CA, USA) experiment protocol. The total RNA was

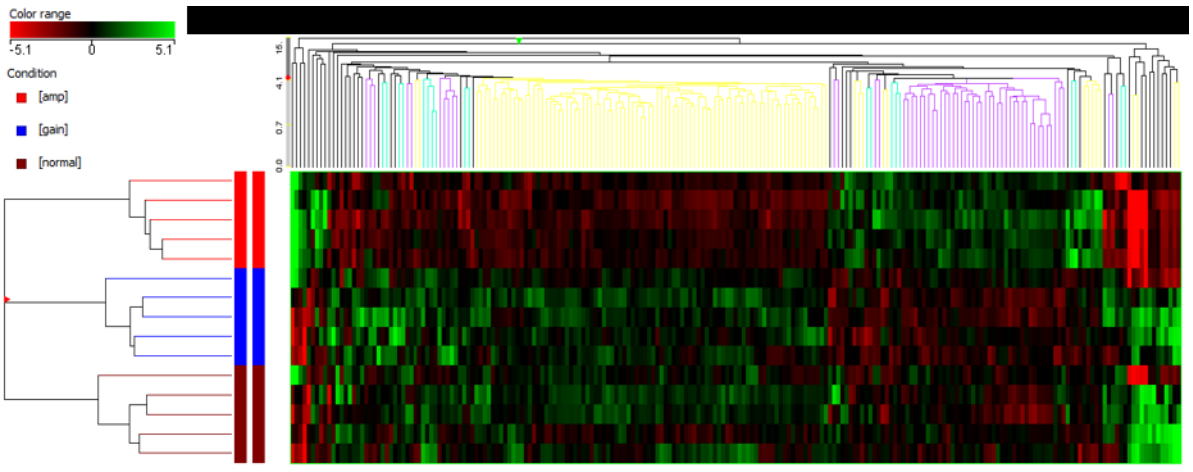

Fig. 1. Fluorescence *in situ* hybridization images of MYCN status in NB nuclei. ***A***, MNA; ***B***, 2p gain; ***C***, normal; ***D***, set of molecular probes.

TABLE 1. Most common described methods for detection of BRAF V600 in clinical setting.

MYCN status	*Age (months)*	*INSS*	*Risk group*	*Sex*
MNA	5.5-72.5 median 25.1	3 (n = 1) 4 (n = 4)	High (N = 5)	♀ (n = 5) ♂ (n = 0)
2p gain	5.5-37.6 median 24.7	1 (n = 2) 3 (n = 2) 4 (n = 1)	Standard (N = 2) Intermediate (N = 2) High (N = 1)	♀ (n = 1) ♂ (n = 4)
Normal	11.2-126.6 median 56.7	2 (n = 1) 3 (n = 2) 4 (n = 2)	Standard (N = 2) Intermediate (N = 1) High (N = 2)	♀ (n = 1) ♂ (n = 4)

amplified from each sample and used as a Cyanine 3 labeled analog of a uridine triphosphate (Cy3-UTP) marker. The slides were scanned using the Agilent Technologies SureScan Microarray Scanner G2600D.

Gene expression and functional analysis

Agilent Feature Extraction v10.7.3.1 software was used for raw data extraction. GeneSpring GX v12.1 software was used for quantile normalization and subsequent processing of the original data. Differences between gene expression in samples from study groups were validated through fold change screening. The significance level of the test was selected as fold changes >1.5 (<-1.5) and *p*-values <0.05.

RESULTS

The hierarchical clustering analysis presented two distinct gene expression patterns, which correspond to cases with and without MNA (Figure 2). Moreover, it demonstrated similarity in the gene expression patterns between NB subtypes with 2p gain and with normal *MYCN* status (Figure 2). The microarray expression analysis was focused mainly on indicating statistically important differences between the MNA and 2p gain NB subtypes, as alterations specific to NB and affected patients' outcomes. Initially, alterations in the expression of 217 transcripts were observed. Genes with *loci* on the X or Y chromosomes were excluded to eliminate sex-dependent differences. Genes and also long non-coding RNAs with log fold change (FC) below 2 were removed. Finally, the expression level of 7 genes was upregulated in MNA NB and 13 genes were downregulated. In Table 2, 20 genes with the highest log FC between the MNA and 2p gain subgroups along with the trends between each NB subtype are presented[17-19].

It was observed a co-occurrence of gene upregulation in the MNA subgroup and downregulation in the normal and 2p gain subgroups (Table 2). These genes were the most downregulated in the 2p gain subgroup. The exception was the *PTGIS* gene, which was upregulated in MNA and in the 2p gain NB subtype in contrast to the normal subgroup (Table 2).

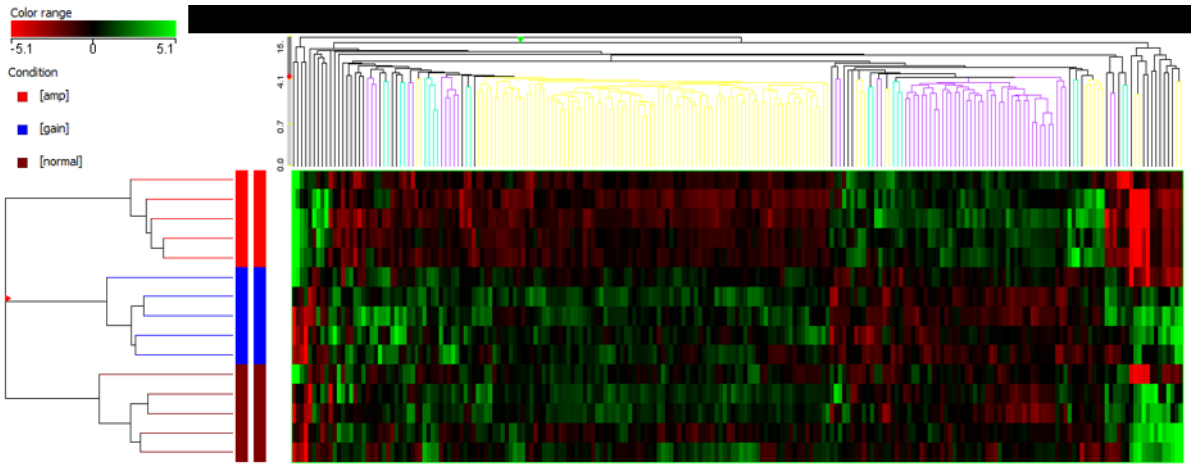

Fig. 2. Hierarchical clustering analysis demonstrating two distinct patterns of relative gene expression in NB with MNA and without (2p gain and normal *MYCN* status).

TABLE 2. Most common described methods for detection of BRAF V600 in clinical setting.

Gene symbol	log FC MNA vs. 2p gain	Trend in NB subtypes			Prognostic marker	Biological function
		MNA vs. 2p gain	MNA vs. normal	2p gain vs. normal		
ESPNL	3.651	↑	↑	↓	None	Actin filament binding
POU4F1	2.945	↑	↑	↓	None	Developing sensory nervous system; promote the growth of cervical tumors
SPINK1	2.484	↑	↑	↓	Renal (UF) and urothelial cancer (F)	Tumor-associated trypsin inhibitor (TATI) is identical to pancreatic secretory trypsin inhibitor encoded by SPINK1 gene; negative regulation of calcium ion import and nitric oxide mediated signal transduction
SLC7A5	2.265	↑	↑	↓	Renal and lung cancer (UF)	Cell differentiation, cellular amino acid metabolic process, nervous system development
DPF3	2.234	↑	↑	↓	None	Transcription regulation; nervous system development
PTGIS	2.126	↑	↑	↑	Renal and urothelial cancer (UF)	Apoptotic signaling pathway; cellular response to hypoxia; negative regulation of inflammatory response; positive regulation of angiogenesis
PEX5L	2.048	↑	↑	↓	None	Regulation of cAMP-mediated signaling
MAMDC2	-2.094	↓	↓	↑	Thyroid cancer (UF)	Proteoglycan
MAEL	-2.128	↓	↓	↑	None	Cell differentiation; cell morphogenesis; gene silencing by RNA; intrinsic apoptotic signaling pathway in response to DNA damage; negative regulation of apoptotic process; negative regulation of transcription
DAPL1	-2.186	↓	↑	↑	Cervical cancer (F)	Apoptotic signaling pathway; cell differentiation; negative regulation of autophagy
SLC12A5	-2.214	↓	↓	↓	Glioma (UF)	K-Cl maintains homeostasis in neurons; dendritic spine development
SIGLEC11	-2.286	↓	↓	↑	None	Cell adhesion; immunosuppressive signaling
ALX1	-2.462	↓	↓	↑	None	Negative regulation of transcription; neural crest cell migration; neural tube closure; positive regulation of epithelial to mesenchymal transition
FNDC9	-2.518	↓	↓	↑	None	Fibronectin; cell adhesion, growth, migration and differentiation
TEKT2	-2.554	↓	↓	↑	Renal cancer (F)	Cilium movement involved in cell motility
MMD2	-2.568	↓	↓	↑	None	Positive regulation of neuron differentiation; positive regulation of Ras protein signal transduction
ASIC2	-2.677	↓	↑	↑	None	Negative regulation of apoptosis; central and peripheral nervous system development
GFRA2	-2.728	↓	↓	↑	None	Neuron survival and differentiation; MAPK cascade; negative regulation of protein autophosphorylation
HYDIN	-3.400	↓	↓	↑	None	Cilia motility; epithelial cell development; ventricular system development
GPM6A	-4.034	↓	↓	↑	None	Differentiation and migration of neuronal stem cells; neuronal plasticity; neurite and filopodia outgrowth

UF-unfavorable; F-favorable.

This finding may indicate that overexpression of this gene is strongly linked to *MYCN* copy number changes.

The second co-occurrence related to genes downregulated in MNA NB. The expression of these genes was higher in the normal and 2p gain subtypes (Table 2). The highest level of expression of these genes was observed in the 2p gain NB. A different pattern was presented by gene *SLC12A5*, which was underexpressed in NB with *MYCN* multiplication – MNA and 2p gain in comparison to normal *MYCN* status (Table 2). This was contrary to two other genes, *DAPL1* and *ASIC2*, that were upregulated in tumors with *MYCN* multiplication, with the highest expression in the 2p gain subgroup (Table 2).

DISCUSSION

Advances in molecular medicine have resulted in an improved ability to predict patients' risk of treatment failure, relapse or death. Molecular stratification of tumors by gene expression profiling was applied to a large number of human cancers. The commercially available expression microarrays aided to create personal treatments for patients at the individual level.

In the present study, differences in gene expression profiles were analyzed to explore the relationship between *MYCN* status and NB biology. The aim was to identify new biomarkers for malignant progression and new molecular therapeutic pathways in two unfavorable genetic subtypes of NB patients – those with MNA and those with 2p gain.

The hierarchical clustering analysis demonstrated two distinct gene expression patterns - one corresponding to cases with MNA and the other to those without MNA (2p gain and normal *MYCN* status). These results are in accordance with studies conducted by other researchers[7,20-22]. However, for the first time a similarity in the gene expression patterns of 2p gain and normal *MYCN* status NB was demonstrated, contrary to MNA. Furthermore, to emphasize the importance of two of the most important genetic alterations affecting NB patient outcomes, a comparison of gene expression between the MNA and 2p gain subgroups was conducted. There were selected the 7 most upregulated genes (*ESPNL, POU4F1, SPINK1, SLC7A5, DPF3, PTGIS, PEX5L*) and the 13 most downregulated genes (*MAMDC2, MAEL, DAPL1, SLC12A5, SIGLEC11, ALX1, FNDC9, TEKT2, MMD2, ASIC2, GFRA2, HYDIN, GPM6A*) in the MNA subgroup to compare with the 2p gain tumors. Among all the genes, 7 (*DAPL1, MAMDC2, PTGIS, SLC7A5, SLC12A5, SPINK1, TEKT2*) have been described as prognostic markers in different types of cancers (Table 2). Moreover, studies undertaken by Budhram-Mahadeo on NB cell lines showed that overexpression of gene *POU4F1*, also known as *Brn-3a,* protects cells from apoptosis[24]. Additionally, it was found that *SPINK1* gene is highly expressed in many cancers and is associated with a poor prognosis[24]. Overexpression of *SPINK1* promotes metastatic behavior especially by matrix metalloproteinase activation as well as by PI3K/AKT and MAPK/ERK signal regulation[25]. In this study, the genes *SLC7A5* and *SLC12A5* were also selected. Glutamine transporters including the solute carrier (SLC) family were found to be cancer-promoting targets and overexpressed in aggressive cancers. Glutamine plays a role in maintaining the activation of mTOR kinase and is required for maintenance of mitochondrial membrane potential and redox control[26,27]. Elorza et al[28] presented that upregulated *SLC7A5* increases mTORC1 activity. Moreover, El Ansari et al[29] found that *SLC7A5* mRNA biosynthesis was associated with the expression of the oncogene *c-MYC* that regulates cellular metabolism and correlated with larger breast tumor size. In addition, the gene *SLC12A5* is a neuronal marker of aggressive cancer stem cells in glioblastoma[30]. Deficiency of *SLC12A5* expression leads to the development of immature neurons with a reduction in active synapses[31]. Li et al[32] demonstrated that *MAEL* overexpression was correlated with cell proliferation, tissue invasion and drug resistance of colorectal cancer cells by inducing epithelial-mesenchymal transition and stem cell properties. Furthermore, higher levels of *ALX1* expression was associated with a poor prognosis, distant metastasis and progression of lung cancer and osteosarcoma[33,34]. A recent study showed that ion channels may play an important role in cancer cell proliferation, apoptosis, invasion, and migration[35]. Zhou et al[36] supported this concept by finding that upregulation of *ASIC2* promotes colorectal cancer invasion and metastasis. An important discovery made by Gu et al[37] suggested that high levels of *GFRA2* expression prompt pancreatic cancer cell growth and chemoresistance through inactivation of suppressor gene *PTEN*. Additionally, Michibata et al[38] and Li et al[39] suggested that suppression of *GPM6A* gene expression in human embryonic stem cells provokes a decrease in the expression of neuroectodermal-associated genes, the number of neural stem cells as well as migration.

Many studies support that *MYCN* is a gene promoting cancer cell growth, motility and invasiveness[40,41]. Moreover, in the literature some genes have been described as direct *MYCN* targets. *MYCN* as a transcription factor upregulates genes involved mainly in cell cycle regula-

tion, cell growth and transcription, in contrast to downregulated genes that take part in processes like apoptosis, nervous system development and cytoskeleton structure[41-45]. Stiglani et al[40] also suggested that MNA mainly drives disruption of neuronal differentiation and reduction of the cell adhesion process involved in tumor invasion and metastasis. Formicola et al[41] proposed that genes repressed in *MYCN* overexpressing cells included *GFRA3*. In this study, another gene for glial cell line-derived neurotrophic factor, *GFRA2,* was identified as downregulated.

CONCLUSIONS

The results of this study support the claim that an increased *MYCN* level promotes cell growth and motility while counteracting differentiation and attachment. These findings are in accordance with previously published data. In the 2p gain and MNA subgroups, genes engaged in negative regulation of apoptosis and autophagy (*ASIC2, DAPL1*) were upregulated in comparison to tumors with normal *MYCN* status. Moreover, *PTGIS*, a gene responsible for downregulating the inflammatory response and for upregulating angiogenesis and the cellular response to hypoxia, was upregulated in 2p gain and also in MNA tumors. Interestingly, it was found that NB tumors with 2p gain, in comparison to MNA and normal *MYCN* subtypes, exhibited a higher expression level of genes involved in cell migration but downregulated genes involved in nervous system development (especially *SLC12A5,* the most downregulated in the 2p gain subgroup). This finding may indicate that 2p gain NB tumors demonstrate more aggressive behavior with a higher tendency to metastasize than MNA cases[46].

The obtained result confirmed that *MYCN* has a significant impact on genome-wide NB gene expression. The studied expression profiles of genetic types of NB identified new candidate genes that may directly relate to *MYCN* status and promote development of more aggressive forms of this pediatric tumor.

Ethical Committee:

The study was conducted according to the Institutional requirements and Helsinki Declaration.

Conflict of Interests:

The author confirms that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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