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# Comparison of the human tumor metastasis gene expression level in neuroblastoma patients with MYCN amplification and 2p gain: Pilot study

Katarzyna Szewczyk

*Department of Medical Genetics, Faculty of Medicine, Jagiellonian University Medical College, Wielicka St. 265, 30-663, Krakow, Poland*

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## 1. Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor during childhood. The amplification of the *MYCN* oncogene (*MYCN*amp) was found in about 25% of NB cases. This alteration is associated with high-risk disease and poor clinical outcome [1,2]. The *MYCN*amp plays an important role in the tumor progression. A large number of *MYCN* copies correlate with invasive and metastatic behavior of NB [3]. Furthermore, the gain of the short arm of chromosome 2 (2p gain) also affects NB cells [4–6]. Moreover, international guidelines for the fluorescence *in situ* hybridization (FISH) examination of NB cells clearly indicate a difference between *MYCN*gain and 2p gain [1]. Resent results suggest that 2p gain is not considered the pre-status of *MYCN*amp [5,7]. Therefore, the main aim in this study was to compare the expression profile of genes engaged in human tumors metastasis in the following two groups of NB patients: 2p gain *vs. MYCN*amp.

## 2. Materials and methods

Between October 2010 and November 2016, 60 infants diagnosed with NB were screened in Children University Hospital in Krakow, Poland, for the *MYCN* oncogene status. The FISH test using the N-MYC (MYCN) amplification probe set was performed on touch-print preparations of the primary tumor tissue according manufacturer's protocol (Cytocell Ltd., Cambridge, UK). In addition, another three unfavorable segmental chromosomal aberrations 1p36 deletion, 11q23 deletion, and 17q gain were studied by FISH. About 12 in 60 (20%) children were diagnosed with *MYCN*amp (3 with heterogeneous form) and 11 (18%) as 2p gain (6 with heterogeneous form). In this study, 6 patients (3 with *MYCN*amp and 3 with 2p gain) were selected based on the following inclusion criteria: alive in remission and homogeneous change of *MYCN* status. The study group included 4 patients over 12th month of life, 4 with the most advanced 4th stage according International Neuroblastoma Staging System (INSS), and 5 having additional chromosomal alterations (Table 1). Patients with NB in Children University Hospital in Krakow are treated according the European International Society of Pediatric Oncology Neuroblastoma Group (SIOPEN) protocols for high-risk as well as low and intermediate risk group.

Total RNA was extracted from deep-frozen (−80 °C) tumors tissues using TRI Reagent™ Solution (Invitrogen, Thermo Fisher Scientific, Waltham, USA). Using a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Waltham, USA), the RNA quantity and purity were determined by measuring UV absorption of the sample. Using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA), total RNA was enzymatically converted to cDNA.

TaqMan Arrays (Applied Biosystems™, Thermo Fisher Scientific, Waltham, USA, cat. nr. 4414098) configured for the set of genes engaged in human tumors metastasis pathways were used for gene expression analysis in the study group. Each 96-well plate contains predefined assays and 4 endogenous controls (*18S, GAPDH, HPRT1,* and *GUSB*). In each single well, 30 ng of cDNA was used. Based on manufacturer's guidelines, the thermal profile was adopted. The fluorescence signal detection was performed with the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA). The comparative $2^{-\Delta\Delta Ct}$ analysis method was used.

Using a two-tailed Student's *t*-test for independent samples (Statistica 12, StatSoft, Poland), statistical significance was determined. A $p$ value of $<0.05$ was considered statistically significant.

*E-mail address:* katarzyna.szewczyk@uj.edu.pl.

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**Table 1**
The clinical characteristics of the selected patients.

| No. | Age at diagnosis (months) | INSS Stage | MYCN status | Other genetic features |
|---|---|---|---|---|
| 1 | 57.43 | 4 | 2p gain | Tetrasomy 2 |
| 2 | 5.10 | 1 | 2p gain | Disomy 2 |
| 3 | 8.73 | 1 | 2p gain | Trisomy 2 |
| 4 | 27.63 | 4 | *MYCN*amp | Tetrasomy 2, del1p36, del11q23, gain 17q |
| 5 | 72.33 | 4 | *MYCN*amp | Tetrasomy2 |
| 6 | 17.23 | 4 | *MYCN*amp | Trisomy 2, del1p36, del11q23 |

## 3. Results

The *HPRT1* gene was excluded from analysis. This gene was the inadequate endogenous control due to the average Ct value over 30. The average Ct values from 3 other control genes (*18S, GAPDH, GUSB*) for 6 samples ranged from 21.2 to 28.8. As it was mentioned, in one sample, the quality of RNA was low (Ct = 28.8), but this sample was not excluded in view of the biological meaning of this very small study group.

The expression level in the set of genes was mostly higher in the *MYCN*amp group (average fold change = 2.88; range: 1.04–7.72 for 65 genes). The highest 7.72 fold change was noted for the *HGF* gene. Only for 12 out of 92 genes expression level was elevated in the 2p gain group (average fold change = 2.37; range: 1.03–7.83) (genes: *CTBP1, CTNNA1, DCC, EPHB2, ERBB2, FGFR4, HRAS, LAMB1, NCAM1, PNN, PECAM,* and *TCF20*). The very low expression level was detected for 15 out of 92 genes: *CCL7, CDH1, ETV4, KISS1, KISS1R, LYPD3, MMP1, MMP3, MMP7, MMP10, PSCA, RET, SERPINB5, TMPRSS4,* and *TSHR* in both groups.

The observed differences in the expression level of selected metastasis genes between studied groups 2p gain *vs. MYCN*amp were significant for *FGFR4* ($p = 0.01$) and *NCAM1* ($p = 0.04$) genes. These genes were higher transcribed in 2p gain NB tumor samples (*FGFR4* fold change = 4.82; *NCAM1* fold change = 7.83).

## 4. Discussion

The *MYCN*amp is one of the most powerful adverse prognostic factors for neuroblastoma [1–3,8]. The multiplication of *MYCN* oncogene copies also appears as 2p gain [4–7]. However, 2p gain was not reported as the independent prognostic factor for neuroblastoma [9]. One way of activating proto-oncogenes is the repetition of DNA segments. It may contribute to enhancing followed gene expression. The clinical significance of increased expression of *MYCN* remains unclear in NB [10]. In addition, it was shown that *MYCN*amp may not directly affect the *MYCN* gene expression in NB patients [11].

The structural chromosomal alteration like 2p gain occurs unexpectedly common among Polish patients diagnosed with NB in comparison to international data (18% *vs.* 6–18%). The meaning of this aberration for NB biology still remains unknown. Therefore, this group of NB patients was the key research theme. NB is the most common solid tumor of childhood. However, the occurrence in Poland is about 70 cases per year, and in Krakow, it is under 10 patients yearly. Consequently, the size of the study group was the largest problem. Nevertheless, a small but very selected group of NB patients was collected.

We reported slight differences in the expression level of genes engaged in human tumors metastasis pathways between our studied groups such as 2p gain *vs. MYCN*amp. The *HGF* gene was the most overexpressed in the *MYCN*amp group. The HGF protein acts as an endogenous ligand for the MET proto-oncogenic receptor of tyrosine kinase and as an angiogenic growth factor. Previous study showed elevated concentrations of HGF in NB patients suggesting an enhanced invasiveness and a correlation with unfavorable genetic markers [12,13].

We found only two significant differences between study groups. There distinctions were concerned with *FGFR4* and *NCAM1* genes whose expression was higher in the 2p gain group. The FGFR4 plays an important role in the regulation of cell proliferation, differentiation and migration, and lipid metabolism. Moreover, expression of *FGFR*4 resulted in decreased cell adhesion. The permanent up-regulation of FGFR4 signaling was observed in rhabdomyosarcoma. The *FGFR4*-activating mutations were also seen in adenocarcinomas and glioblastomas. In hepatocellular carcinoma, *FGFR4* expression correlated with worse prognosis via stimulation of hepatocyte proliferation. In astrocytomas, *FGFR4* overexpression was correlated with advanced tumor stages and short survival, as well as prostate and thyroid cancers [14]. In colorectal cancer, the *FGFR4* gene upregulation acts as a radioresistance factor. The NB tumor cells have been shown to express the FGFR4 ligand and FGFR family members [15]. There is no confirmed role of the *FGFR4* gene in biology and development of the NB cancer cells, but there are indications that it may be a potential prognostic factor. Moreover, latest studies have shown an association between the *FGFR4* polymorphism (Gly388Arg) and increased incidence of NB. This polymorphism has been reported as an important factor in decreasing survival rates, treatment resistance, and more aggressive disease in a variety of malignancies [14,16]. Megioni et al. found that microRNAs regulating gene expression were differentially expressed in NB with or without *MYCN*amp, and these micromolecules were also involved in the *FGFR* pathway [17]. Moreover, possible anticancer therapies were suggested based on FGFR4 inhibition by antibody or short hairpin (sh)RNA blocking [14].

The NCAM1 protein is a member of the immunoglobulin superfamily involved in cell-to-cell and cell-matrix interactions during development and differentiation. The NCAM1 has also been shown to be involved in development of the nervous system. The *NCAM1* gene is widely expressed in neuroectodermal tumors such as neuroblastoma [18]. A comparative study on the expression of NCAM isoforms in NB tissues has indicated an association between an isoform and tumor differentiation status and metastatic potential [19]. Moreover, NB tumor cells express polySia-NCAM that correlates with aggressive disease and poor clinical outcome. Therefore, enzymes engaged in polySia-NCAM biosynthesis and NCAM protein are considered as druggable targets [20,21]. In addition, in the mouse model of the pancreatic tumor, NCAM formed a complex with FGFR4. This complex was required for neurite outgrowth and cell-matrix attachment of the tumor cells. Authors suggested that this dependency explains the metastasis formation [14].

## 5. Conclusions

The *MYCN*amp remains the most unfavorable prognostic factor in NB. However, there is still limited knowledge about effects of the *MYCN* oncogene and multiplication of its chromosomal *locus* 2p24 on the NB tumor cell biology. Our research sheds new light on the

role and mechanism of 2p gain in the pathogenesis of neuroblastoma. We indicated two potentially important genetic factors, but additional studies are required to confirm mentioned associations. The study group should be enriched about new participants. The research data about the *FGFR4* expression level should be correlated with the mentioned polymorphism and clinical features. The microarray expression analysis may indicate new connection pathways.

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