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EGFR, *PIK3CA* and *PTEN* gene status and their protein product expression in neuroblastic tumours

Ewa Izycka-Swieszewska[1], Magdalena Brzeskwiniewicz[2], Agnieszka Wozniak[3], Elzbieta Drozynska[4], Wieslawa Grajkowska[5,6], Danuta Perek[7], Anna Balcerska[4], Teresa Klepacka[8], Janusz Limon[2]

[1]Department of Pathomorphology, [2]Department of Biology and Genetics, Medical University of Gdansk, Poland, [3]Laboratory of Experimental Oncology, Department of General Medical Oncology, Catholic University of Leuven, University Hospitals, Leuven, Belgium, [4]Department of Paediatrics, Haematology, Oncology and Endocrinology, Medical University of Gdansk, Poland, [5]Department of Pathology, Children's Health Memorial Institute, Warsaw, Poland, [6]Department of Experimental and Clinical Neuropathology, M. Mossakowski Medical Research Centre, Warsaw, Poland, [7]Department of Oncology, Children's Health Memorial Institute, Warsaw, Poland, [8]Department of Pathology, Institute of Mother and Child, Warsaw, Poland

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Abstract

PI3K/AKT/mTOR pathway signalling is often upregulated in cancer, usually by the constitutional activation of growth factor receptors, amplification or mutation of PIK3CA and loss of tumour suppressor PTEN function. The way of PI3K/AKT/mTOR pathway activation in neuroblastoma (NB) is not well established.

The study was performed on paraffin-embedded tissue sections from 106 patients with NB. The aim of the study was to analyse the mutational status of EGFR (exons 18-21), PIK3CA (exons 5, 6, 10 and 21) and PTEN (all exons) genes, as well as to assess expression of their protein products by immunohistochemistry.

A novel mutation in exon 5 of PIK3CA, c.931 A>G (p.I311V), in two infantile tumours (2.7%) was identified. In addition some polymorphisms were found in all examined genes, including a novel one, c.285 A>T in PTEN. Polymorphism PIK3CA c.1060-17 C>A was significantly more frequent in extra-adrenal tumours. Polymorphism PIK3CA c.1145+54 A>G showed a tendency to be more frequent in children older than 18 months and in extra-adrenal tumours. Expression of EGFR was present in 95% of cases, PI3Kp110 in 92% of tumours and PTEN in all tumours (low in 39%) and did not correlate with the genetic alterations. EGFR and PTEN expression showed an association with tumour differentiation.

Mutations in the EGFR, PIK3CA and PTEN genes are infrequent in neuroblastoma. Both newly detected mutations in exon 5 of PIK3CA occurred in very low risk neuroblastic tumours in infants. EGFR, PI3Kp110 and PTEN expression is a common feature of NB.

Key words: neuroblastoma, EGFR, PIK3CA, PTEN, mutation, PI3K/AKT/mTOR pathway, protein expression.

Introduction

Neuroblastic tumours (NB) create a heterogeneous group of neural embryonal neoplasms developing in the adrenal medulla and sympathetic ganglia [12,15]. Neuroblastic tumours are the most common extracranial tumours of childhood. The prognosis of patients and therapeutic approach depend on widely accepted risk factors including age, stage, tumour histology, as well as several genetic aberrations [3,12,27].

Communicating author:
Ewa Izycka-Swieszewska, MD, PhD, Department of Pathomorphology, Medical University of Gdańsk, 7 Debinki St., 80-211 Gdansk, e-mail: eczis@wp.pl

However, insight into the molecular alterations and pathways involved in NB biology is necessary for the development of new targeted therapies [12,29].

One of the most important signalling pathways in cancer is PI3K/AKT/mTOR, which plays a complex role in cell survival, proliferation and differentiation [9,11,30]. Deregulation of this pathway is connected with oncogenic transformation, cancer progression and metastasis [2,13]. In parallel, selective inhibitors against members of this pathway are under intense investigation [7,13,18]. The main mechanisms of abnormal activation of the PI3K/AKT pathway in cancer include constitutional activation of growth factor receptors (EGFR, IGF-IR, HER2), alterations of *PIK3CA* and loss of tumour suppressor *PTEN* function [8, 23-25]. The pathological activation of growth factor receptors such as EGFR results mainly from gene amplification, mutation and/or protein overexpression. EGFR alterations at the genetic and protein level tend to have prognostic and predictive significance in several types of neoplasms [18,26,31]. The *PIK3CA* gene, which encodes the catalytic alpha subunit (p110) of PI3K, is mutated or amplified in a broad spectrum of tumours [8,11,24]. *PTEN* changes, found in 30% of human cancers, include its loss, inactivating point mutations or lowered expression [23,25,28].

The way of PI3K/AKT/mTOR pathway activation in neuroblastoma (NB) is not well established. In the present study we performed an analysis of the *PIK3CA*, *PTEN* and *EGFR* genes and their protein products in a large series of clinical NB tumour samples.

Material and methods

Material

The study was performed on archival material of routinely processed paraffin-embedded tumour sections taken at the surgical biopsy or tumour resection. Tumour tissue was collected from 106 neuroblastic tumours, obtained from children treated in Polish paediatric oncological centres. Representative tumour samples were chosen for immunohistochemical and molecular analyses. The patho-clinical data included: patients' age, tumour stage, primary tumour localization, tumour histology, *NMYC* status and patients' survival.

Patients' age ranged from 1 to 169 months (median 30 months), including 40 children below 18 months of age. The group consisted of: 10 tumours in stage I, 13 in stage II, 34 in stage III, 44 in stage IV and 5 in stage IVs. Primary tumour localization was adrenal in 55 cases, abdominal extra-adrenal in 26, and other (mediastinal, sacral, cervical) in 23 patients. Histologically, 77 Schwannian stroma-poor NB, 20 Schwannian stromarich NB, and 9 Schwannian stroma-predominant NB were diagnosed. *NMYC* was amplified in 25 cases.

Patients' follow-up ranged from 3 to 152 months (last observation April 2010) and during this time 35 children died.

Molecular studies

For DNA extraction 5-µm sections were cut from each tumour sample from paraffin blocks and genomic DNA was isolated using the standard protocol with proteinase K digestion, phenol-chloroform extraction and ethanol precipitation. The somatic status of *EGFR* (exons 18-21), *PIK3CA* (exons 5, 6, 10, 21) and *PTEN* (all exons) was analysed using polymerase chain reaction (PCR) and direct sequencing. Specific primers were designed using the Primer3 web tool (http://frodo.wi.mit.edu/primer3) and their sequences and PCR cycling parameters are available on request. Obtained sequences were compared with reference transcript sequences available online (www.ensembl.org – *EGFR*: ENST00000275493, *PIK3CA*: ENST00000263967, PTEN: ENST00000371953) using Sequencer v.4.7 (Gene Codes Corporation, USA). To predict the possible impact of an amino acid substitution on the structure and function of the protein, the PolyPhen web-based tool was used (http://genetics.bwh.harvard.edu/pph).

FISH analysis of *EGFR* copy number was carried out on sections from 80 cases. Following deparaffinization and hydration, pre-treatment and enzyme digestion were done using the SPoT-Light Tissue Pretreatment Kit according to the manufacturer's recommendations (Invitrogen, USA). Hybridization with the Vysis EGFR-SpectrumOrange (SO)/CEP7-SpectrumGreen (SG) probe set was performed following the manufacturer's protocol (Abbott Molecular, USA). Slides were counterstained with 0.1 µmol/L 4,6-diamidino-2-phenylindole (DAPI) in an antifade solution and viewed under a fluorescence microscope equipped with SO/SG/DAPI set of filters (Axioskop, Zeiss). Images were obtained and analysed using CytoVysion Ultra System digital image analysis software (Applying Imaging International Ltd). Signal copy numbers were counted from 40 to 100 non-overlapping nuclei from a minimum of three different randomly chosen tumour areas. The presence of gene

clusters (≥ 4 spots) or at least 15 copies in ≥ 10 tumour cells was considered amplification.

Immunohistochemical analysis

Immunohistochemistry was performed on tissue microarrays (TMA) constructed using Tissue-Tek® Quick-Ray™ Tissue Microarray System (Sakura Finetek USA Inc) according to the manufacturer's instructions. One or two 5 mm core biopsies were obtained for each tumour and mounted into recipient blocks. The following antibodies were used for immunohistochemical evaluation: EGFR (Novocastra NCL-L-EGFR, mc., Clone EGFR.113; 1 : 10), PI3Kp110 (SCBT mouse PI3Kp110 sc-7177, pc., 1 : 100) and PTEN (Novocastra, mouse anti-hu NCL-PTEN, mc., 1 : 100) with appropriate positive and negative controls included. The standard protocol with heat-induced antigen retrieval in citrate buffer was performed. Next the slides were incubated with the primary antibody for 30 minutes at room temperature. After that the visualization system En Vision and DAB (DAKO) with haematoxylin staining was used.

EGFR, PI3Kp110 and PTEN expression was assessed in the neuroblastic cell component of tumours in a descriptive and semi-quantitative method. The expression was categorized as negative, low or high, based on the percentage of positive cells and staining intensity as previously described [6].

Statistical analysis

For univariate analysis, Fisher's exact test, Chi-square test and Mann-Whitney test were used to compare categorical and continuous variables respectively. The relation between continuous and ordinal variables was analysed with Spearman's rank correlation. The inter-observer agreement in immunohistochemistry assessment was measured with the kappa statistic (κ). Survival analysis was performed with the Kaplan-Meier method and survival between groups was compared using the log-rank test. Data were expressed as median and range. The value of $p < 0.05$ was interpreted as statistically significant. Analyses were performed with the software package STATISTICA (StatSoft Inc, 2007, USA).

Results

Mutation analysis

In two samples of 73 (2.7%) available for analysis a novel mutation in exon 5 of *PIK3CA* (c.931 A>G; p.I311V) was found (Fig. 1). This alteration was not identified in normal tissue from adrenal gland of the same patients, proving the somatic origin of the mutation. However, *in silico* modelling shows that this change probably does not have a phenotypic effect on protein. Both children with that mutation were infants with very low-risk tumours. One mutation occurred in an adrenal NB stage I in a 5-month old girl, while the second occurred in an adrenal NB stage IVs in a 3-month old boy. Both tumours were non-*NMYC*-amplified, poorly differentiated Schwannian stroma-poor neuroblastomas. The first patient underwent radical surgery and the second received chemotherapy with the protocol European NB 99.2. The children are alive and free of symptoms with a long period of follow-up (72 and 75 months since diagnosis).

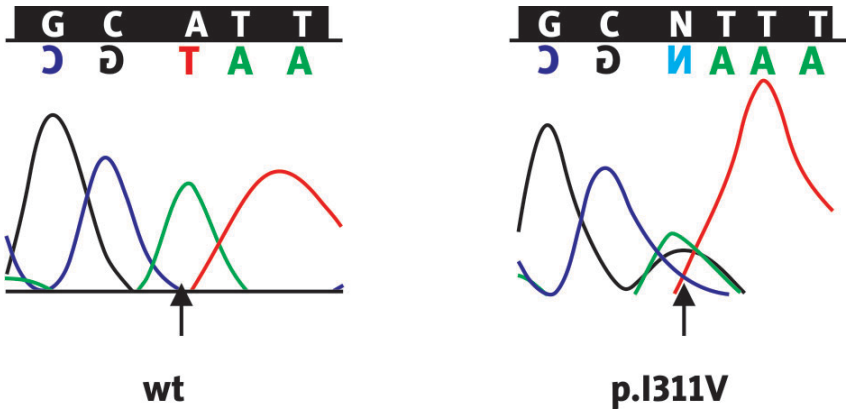

Fig. 1. Fragment of sequence with identified *PIK3CA* p.I311V mutation.

We did not find any mutations in the *EGFR* and *PTEN* genes in the examined group.

However, six polymorphisms were identified: three in the *EGFR* gene (c.2184+19 G>A, c.2361 G>A and c.2508 C>T), two in the *PIK3CA* gene (c.1060-17 C>A and c.1145+54 A>G), and one novel alteration in the *PTEN* gene (c.285 A>T). All of the *EGFR* and *PIK3CA* polymorphisms represent a previously identified SNP based on a query of the dbSNP (Table I). Three of the six polymorphisms are located in non-coding regions and three are located in exons, but they do not lead to alteration in the sequence of amino acids.

The NB case in stage IVs with *PIK3CA* mutation also showed polymorphisms c.2184+19 G>A and c.2361 G>A in the *EGFR* gene and c.285 A>T in the *PTEN* gene.

In addition, while sequencing exon 10 of the *PIK3CA* gene, we also found three alterations: c.1634 A>C, c.1658 G>C and c.1659del1 in all the cases. Based on the literature we concluded that those alterations were artefacts created by interferences from the sequence homologue located on chromosome 22 [21].

In the FISH study, the average number of *EGFR* signals per nucleus in tumour tissue ranged from 1.57 to 5.86, while the EGFR/CEP7 ratio ranged from 0.82 to 1.67, with no evidence of gene amplification.

Immunohistochemistry

EGFR membrano-cytoplasmic expression was found in almost all tumours, with a high intensity in 78% of cases. EGFR expression differed between histopathological groups ($p = 0.003$) and was significantly correlated with tumour histology (Rs = 0.36, $p < 0.001$), showing an increase parallel to tumour maturity (Fig. 2A-B).

PI3Kp110 cytoplasmic expression was found in 98 cases (92%). Low expression concerned 46 and high expression 52 cases. No correlations with patho-clinical data were revealed. The expression was present in undifferentiated neuroblasts as well as in ganglion cells. In both tumours with *PI3KCA* mutations expression was present – low in one and high in the second case (Fig. 2C-D).

PTEN expression was nuclear. It was revealed in all cases, but it was low in 41 (39%) and high in 65 (61%) cases (Fig. 2E-F). The expression depended on histology, decreasing in maturing neuroblasts. High PTEN expression was found more frequently in Schwannian stroma-poor tumours (52 vs. 13) than low PTEN (22 vs. 16) ($p = 0.01$).

No correlation was found for PTEN vs. EGFR, PTEN vs. PI3K, or PI3K vs. PTEN expression. Also PI3K, PTEN and EGFR protein expression did not correlate with gene polymorphisms.

Table I. Frequency of identified polymorphisms in selected genes

Localization	Polymorphism	dbSNP* ID	Alteration in amino acid sequence	Frequency in study group (%)		Frequency in Caucasian population (dbSNP*) [%]	
EGFR				htz	hom	htz	hom
1. Intron 18	c. 2184+19 G>A	rs17337107	Non-coding region	10 (16.7)	0	3.3	0
2. Exon 20	c. 2361 G>A	rs1050171	p.Q787Q	26 (46.4)	15 (26.8)	46.7	18.3
3. Exon 21	c. 2508 C>T	rs17290559	p.R836R	2 (4.7)	0	16.7	0
PIK3CA							
4. Intron 5	c. 1060-17 C>A	rs2699896	Non-coding region	20 (32.8)	20 (32.8)	37.6	39.6
5. Intron 6	c. 1145+54 A>G	rs3729679	Non-coding region	19 (31.1)	17 (27.9)	38.6	33.7
PTEN							
6. Exon 5	c. 285 A>T	nd	p.P95P	4 (9.5)	0	nd	nd

**dbSNP (http://www.ncbi.nlm.nih.gov/SNP), htz – heterozygous, hom – homozygous, nd – no data*

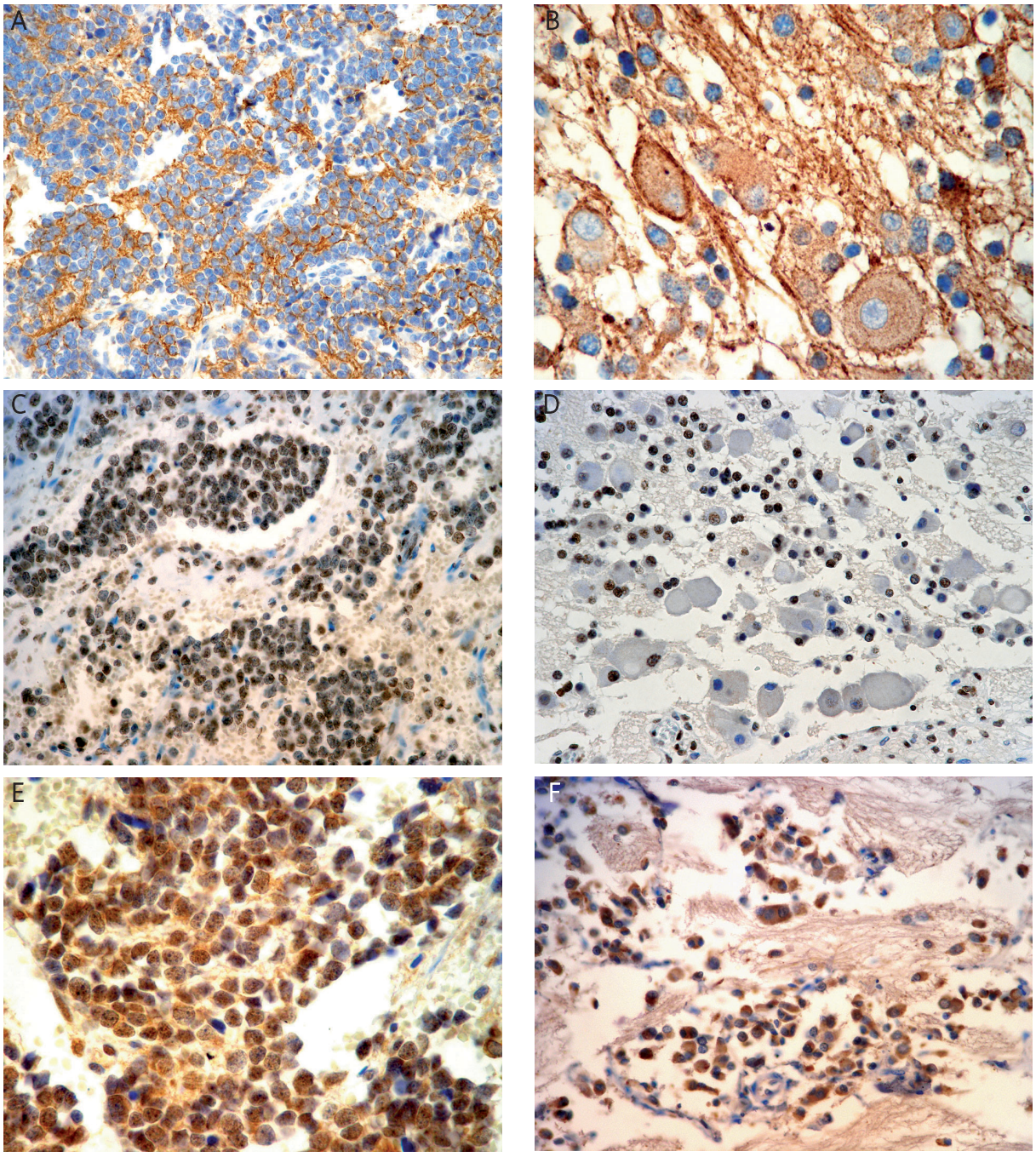

Fig. 2. Tissue expression of analysed proteins. **A)** Membranous EGFR expression in poorly differentiated NB (× 200). **B)** High membrano-cytoplasmic EGFR immunoreactivity in differentiating NB (× 400). **C)** High nuclear PTEN reactivity (× 200). **D)** PTEN expression decreasing in maturing neuroblasts/ganglion cells (× 200). **E)** PI3K p110-positive, poorly differentiated NB (× 400). **F)** Cytoplasmic PI3K p110 expression in differentiating neuroblasts (× 200).

Statistical analysis of polymorphisms

In the analysis of the presence of polymorphisms vs. the unchanged gene, no differences in patho-clinical features were revealed for *EGFR* c.2184+19 G>A vs. EGFR or *EGFR* c.2361 G>A vs. EGFR. On the other hand, *PIK3CA* c.1060-17 C>A was more frequent in extra-adrenal tumours ($p = 0.005$). This polymorphism showed a tendency to be more frequent in Schwannian stroma-rich and predominant tumours than in the stroma-poor group ($p = 0.08$). Polymorphism *PIK3CA* c.1145+54 A>G showed a tendency to significantly more frequent occurrence in children older than 18 m and in extra-adrenal tumours ($p = 0.065$ and $p = 0.052$ respectively).

Moreover, no associations were found between patient survival and presence of *EGFR, PTEN* and *PIK3CA* polymorphisms.

Discussion

Neuroblastic tumours constitute a complex neoplastic disease of the autonomic nervous system, in which interactions of effects from multiple genetic alterations might be needed for tumorigenesis [12]. The changes observed in the NB genetic profile are heterogeneous and no key alteration connected with cancerogenesis, or with disease progression mechanisms, has been established [12,29]. Familial NB cases carry an *ALK1* or *PHOX2B* mutation, but this reflects only a small group of patients [15]. On the other hand, the most common alterations, such as *NMYC* amplification, additional unbalanced copy of 17q, and deletions of 1p, 11q and 14q, appear at different levels of oncogenesis and stages of NB [3,12,29]. The cancer genes most commonly altered in adult tumours are rarely aberrant in paediatric tumours, including NB [12]. However, several reports concerning the role of the PI3K/AKT/mTOR pathway in NB have appeared in recent years [1,19,22]. We studied the status of *EGFR*, *PIK3CA* and *PTEN* using both direct sequencing and immunohistochemistry to assess the mutational status and protein expression in a large series of NB with available clinical data.

EGFR overexpression with or without gene alterations observed in many types of carcinomas and glioblastoma is often associated with tumour progression and poor outcome [18,26,31]. Furthermore, *EGFR* gene copy number and mutations in the tyrosine kinase domain modulate the response to its selective inhibitors in cancer patients [7,18]. The anti-EGFR target therapy creates a promising approach for NB patients, especially those with advanced refractory tumours [7,22]. In our study, we did not detect mutations or amplification of *EGFR*. In the recent report, no mutations of this gene have been revealed in NB cell lines either [22]. We identified three polymorphisms in *EGFR*: one within intron 19 (c.2184+19 G>A), one within exon 20 (c.2361 G>A), and one within exon 21 (c.2508 C>T). The latter two were silent changes that did not change the amino acid sequence of the protein. There were no significant differences in the frequency of c.2184+19 G>A and c.2361 G>A polymorphisms between healthy individuals (of European descent) and those with neuroblastoma from our group (Table I). Also, a similar frequency of the above-mentioned polymorphism was found in other tumour types [26]. EGFR membrano-cytoplasmic expression was a common feature of our NB group and showed increasing intensity parallel to neuroblastic cell maturation. There were no associations between EGFR expression and gene polymorphisms either.

The *PIK3CA* gene encodes a catalytic p110 alpha subunit of PI3-kinase [8,24]. Its mutations, leading to constitutive kinase activity, are present in up to 30% of different tumour types. PI3K targeting creates a promising modern method of cancer therapy [11,13]. 80% of known *PIK3CA* mutations concern exons 10 and 21, encoding the kinase activity domain [5,9,24]. The others occur in exons 5 and 6, which encode the C2 region of the protein [5,11]. The mutations in exon 5 are found in 5% of different tumours, but are more common in glioblastoma [5,11]. The *PIK3CA* gene in neuroblastoma was previously examined in only one study on 42 tumour samples and 27 cell lines, performed by Dam *et al*. [4]. These authors found two mutations (2.9% of cases) with unknown functionality. One mutation was located in exon 10 (R524M), with no confirmation of whether it was somatic, and the second was a somatic mutation within the kinase domain (E982D) [4]. We identified for the first time the somatic mutation in exon 5 of *PIK3CA* c.931 A>G (p.I311V), which occurred in two cases (2.7% of the group), constituting 6.4% of children under 18 months of age. Both mutations occurred in infantile tumours classified in a very low pre-treatment risk group. One tumour in stage IVs had parallel polymorphisms in *EGFR* and *PTEN*. The functional significance of p.I311V has not yet been established but *in silico* modelling shows low probability of having a phenotypic effect on PI3Kp110 protein.

In mutational analysis, two linked polymorphisms of the *PIK3CA* gene were identified – one located in intron 5 (c.1060-17 C>A) and the second located in intron 6 (c.1145+54 A>G). There were no significant differences in the frequency of identified polymorphisms between the healthy individuals (of European descent) and those in our samples (Table I). A significant patho-clinical association was found in the PI3K polymorphism *PIK3CA* c.1060-17 C>A, which was more frequent in extra-adrenal tumours ($p = 0.005$). The polymorphism *PIK3CA* c.1145+54 A>G showed a tendency to be more frequent in children older than 18 months and in extra-adrenal tumours. Moreover, while sequencing for mutations in selected exons of the *PIK3CA* gene, we found an alteration at nucleotide c.1634 A>C (p.E545A) in all the cases, which coexisted with two other alterations: c.1658 G>C and c.1659del1. In addition, we found in exon 10 of *PIK3CA* in all tested cases three alterations: c.1634 A>C, c.1658 G>C and c.1659del1. Qiu *et al*. proved the presence of two genomic loci in chromosome 22 (cat eye syndrome region) and in chromosome 16, that contain highly homologous sequences to *PIK3CA* exons 10, 12-14 and partial exon 11 (97% similarity) [21]. Therefore we concluded that the above-mentioned alterations were artefacts created by additional amplification of homologue sequences.

The impact of alterations of PI3K tissue expression on PI3K signalling and disease remains to be established [9]. There are no data about tissue expression of PI3K in NB tissue. In our series, PI3K p110 was expressed in neuroblastic cells in a majority of tumour samples, with no associations with patho-clinical data.

About 30% of human tumours, mostly breast, ovary, brain and prostate cancer, are associated with genetic alterations or deregulated *PTEN* expression [23,25,28]. The signature of *PTEN* loss is associated with a poor outcome in cancer patients [23]. In our study we analysed all coding sequences of the *PTEN* gene at the DNA level, finding no mutations. However, in 9.5% of samples, we detected a novel heterozygous, silent polymorphism in exon 5: c.285 A>T (p.P95P). Previously, Moritake *et al*. [14] investigated 15 NBT cell lines to search for mutations in the *PTEN* gene using SSCP analysis. Only one of their analysed cell lines, established from a patient during a disease recurrence, showed an alteration in exon 7. Munoz *et al*. [16], in their group of 45 NB tumours and 12 cell lines, found a homozygous deletion in exon 2 in two cases. All these results together with our findings suggest that alterations of the *PTEN* gene are infrequent in NB.

The prognostic role of PTEN protein status in cancer is controversial [23,28]. In fact, the loss of nuclear but not cytoplasmic PTEN seems to correlate with the progression of certain tumours [17]. In our study PTEN nuclear expression characterized all the cases, with no correlation with patient survival. The only patho-clinical association was more frequent low expression in Schwannian stroma-rich and predominant tumours than in stroma-poor NB. One of various PTEN functions is control of neuronal differentiation, where PTEN interacts with NGF signalling and regulates the transition of differentiating neuroblasts into post-mitotic neurons [10,17]. Interestingly, Qiao *et al*. examined 24 NB cases and reported a decreased level of PTEN protein expression in undifferentiated tumours [20] when compared with matured, well-differentiated tumours. They proposed the role of lowered PTEN in NB progression.

Our results show that mutations in the *EGFR*, *PIK3CA* and *PTEN* genes are infrequent in neuroblastoma. The few available reports share our results indicating the rarity of genetic changes in *PIK3CA*, *EGFR* and *PTEN* genes in NB. The presence of a new *PIK3CA* mutation in two tumours in infants from a very low risk group suggests the possible involvement in pathogenesis of an NB subset. Expression of EGFR, PI3Kp110 and PTEN is a common feature of NB.

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