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Expression and Methylation Pattern of p16 in Neuroblastoma Tumorigenesis

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Abstract Understanding migration, population and differentiation of primordial neural crest cells will help in evolving biology of neuroblastoma. P16 is a tumour suppressor gene contributing in cell cycle arrest as cyclin dependent kinase inhibitor. Methylation is an important mechanism for silencing tumor suppressor genes. The aim of this study was to evaluate the role of p16 and its methylation pattern in neuroblastoma tumorigenesis. This study included 23 cases (11 male; 12 female) and 31 samples from archival paraffin embedded tissues. P16 was studied in 5 samples of normal adrenal medullar tissue, 5 samples of adrenal tissue including blastic rests, 5 samples of neuroblastoma in situ tissue and in 8 samples of neuroblastoma tissues primary and after chemotherapy in each group. The adrenal gland tissues were obtained from paediatric autopsy cases. Expression of p16 was searched by immunohistochemistry. Methylation specific PCR was used to detect the methylation rate of p16. The age range of autopsy cases was between 20 weeks of foetal age and 36 months of infant age. The mean age of neuroblastoma cases was 45 months. P16 expression was positive in normal adrenal tissues, in one of 5 samples of adrenal blastic rest tissue and in all of samples of after chemotherapy; while no expression was observed in neuroblastoma and neuroblastoma in situ tissues. P16 methylation was observed in samples of neuroblastoma in situ and primary neuroblastoma tissues. Our results suggest that p16 and its methylation seems to play role in neuroblastoma tumorigenesis and in the migration, population and differentiation of primordial neural crest cells. Inhibitors of DNA methylation may provide a useful tool for restoring p16 activity in neuroblastoma treatment.

Keywords Neuroblastoma · p16 · Methylation

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Introduction

Revealing the secrets of the mechanisms underlying the migration, population and differentiation of primordial neural crest cells will help in understanding of evolving biology of neuroblastoma (NB). The best characterized genetic aberration in NB is MYCN amplification, which has been clearly related to prognosis. Several potential tumor-suppressor genes have been described as frequently silenced by hypermethylation in pediatric NB [1–3]. These studies indicated that hypermethylation of tumor-suppressor genes is related to NB evolution. These epigenetic alterations could be used as a marker of the disease, and also as potential therapeutic targets [1]. But these epigenetic changes have not been studied in a NB tumorigenesis model including blastic rest and NB in situ tissues.

P16 is a tumour suppressor gene contributing in cell cycle arrest as a member of cyclin dependent kinase inhibitors family. P16 regulates the G1-S cell cycle transition by inhibiting the cyclin D-cyclin-dependent kinase (CDK)4/CDK6-mediated phosphorylation of retinoblastoma protein [4]. It is affected in a variety of human tumors. Senescence response to telomere dysfunction is reversible and is maintained primarily by p53. However, p16 provides a dominant second barrier to the unlimited

growth of human cells [5]. In contrast to normal cells, the function of p16 gene or its downstream mediators is frequently deregulated in many types of human cancer [6]. Up-regulated p16 expression was found to be associated with advanced stage disease of NB and thus it might represent a prognostic feature of NB [4].

Methylation is an important mechanism for silencing of tumor suppressor genes. Methylation of promoter regions of CpG-rich sites is an important mechanism for silencing of tumor suppressor genes. P16 was found to be methylated in 2% of 51 medulloblastoma cases [7] Hypermethylation of the p16 5′ CpG island is not a frequent event in HR-HPV-positive cervical carcinomas [8]. Aberrant promoter methylation of p16 was found to be present in 52% of pancreatic ductal carcinoma cases [9]. Liu et al. suggested an association between tobacco smoking and an increased incidence of aberrant promoter methylation of the p16 and MGMT genes in non-small cell lung cancers [10].

We hypothesize that methylation of tumor suppressor genes, which especially affects cell cycle would be effective on NB tumorigenesis. We aimed to evaluate the role of tumour suppressor gene p16 and its methylation pattern in NB tumorigenesis model in order to support our hypothesis.

Materials and Method

This study was approved by the Local Research Ethic Committee of Dr Behcet Uz Children's Research Hospital, Izmir, Turkey.

Patients and Specimens

P16 was studied in 5 samples of normal adrenal medullar tissue, 5 adrenal tissue including medullar blastic rests, 5 NB in situ in adrenal tissue and in 8 NB patient tissues before and after chemotherapy. The adrenal tissues were obtained from paediatric autopsy cases. The adrenals were removed during necropsies and fixed in formalin and embedded in paraffin. Haematoxylin and eosin stained and mounted archival slides of 250 bilateral adrenals (4 slides for each case) were re-evaluated in order to scan for neuroblast clusters. The adrenal tissues were classified according to their cell characteristics and distribution. A distinct adrenal medulla is not present in foetus, but it is almost formed at birth. In foetus chromaffin cells, however are haphazardly scattered throughout the foetal cortex. Neuroblastic cells from the neural crest, which have migrated through the cortex, were not mistaken for NB [11]. Area of neuroblast clusters in the medulla but not infiltrating cortex were considered as medullar blastic rests. Normal ratio of cortex to medulla was not changed. Abundant area of neuroblast clusters forming mass in medulla and/or infiltrating cortex but not outside adrenal were considered as NB in situ. P16 expression was searched by immunohistochemistry and methylation specific PCR (MSPCR) was used to detect the methylation rate of p16 in the samples by DNA bisulphide modification following DNA extraction.

Immunohistochemical Staining

Immunohistochemical staining for p16 protein was performed on 3 μm sections cut from formalin fixed tissues embedded in paraffin. After the sections were deparaffinized in xylol, fixed in ethanol, rehydrated, endogenous peroxidase activity was quenched in 3% H2O2 in distilled water for 10 min at room temperature and boiled in citrate buffer (pH 6) in microwave at 400 W. After treatment with blocking solution for 5 min, the slides were incubated with rabbit antihuman p16 Ab (C-20; Santa Cruz Biotechnology) at 1.0 g/ml for 1 h at room temperature at a dilution of 1:100. Biotin-streptavidin, horse reddish peroxidase method as described by the manufacturer was used for visualization (universal kit DBS, KP50L). The Ab-antigen complexes were visualized with diaminobenzidine, using hematoxylin as the counterstain. Nuclear expression more than 10% of the cells was considered as positive for p16 [12].

DNA Isolation, Bisulphite Modification, and Methylation Analysis

MSPCR was used to detect the methylation rate of p16 in the cases by DNA bisulphide modification following DNA extraction. After the excess paraffin on the surface of the block was cut away, 1 mm3 tissue was removed from paraffin block by core biopsy needle. The area was chosen from the demonstrating area matched from the slides. Nearly the entire sample is composed of tumor tissue. The tissues were fixed in buffered formalin (10%, diluted with PBS). Genomic DNA was extracted from these paraffin-embedded tissues according to manufacturer's instruction) (EX-WAX DNA extraction kit for paraffin-embedded tissue, Chemicon, S4530). DNA quality was tested by spectrophotometer at 260/280 nm. Calculation of DNA for each case needed for PCR was done according to Spectrophotometric results (3–7 μL). Sodium Bisulfite conversion was done according to manufacturer's instruction. (Chemicon, S7824, Cp Genome Fast DNA Modification kit). All unmethylated cytosines are deaminated and sulfonated converting them to uracils, while 5-methylcytosines remained unaltered. [19]. The PCR primer for p16 was designed to specifically amplify the promoter region of this gene (Chemicon CpG WIZ p16 Amplification kit S7800). MSPCR contains three oligonucleotide primer sets The M primer set (methylated) detected the product if

the promoter of the gene is methylated. The U Primer set (unmethylated) annealed to unmethylated DNA. W primer set (wild type) served as a control annealing to methylated or unmethylated DNA studied without chemical modification. For each case three sets were studied with hot start PCR. Three master mixes were prepared each including dNTP mix, PCR buffer, primer (U or M or W), taq polimerase (Platinium Taq Polymerase, Invitrogen, 10966-018) and distilled water according to manufacturer's instruction. Amplification was performed as follows: Denaturation of Taq polymerase for 2 min at 94°C and 35 cycles as denaturation for 45 s at 94°C, annealing for 45 s at 60°C and extending for 60 s at 72°C at thermal cycler (Biorad Mycycler). PCR products were visualized by horizontal electrophoresis on a 2% agarose gel with ethidium bromide. Positive controls were Methylated and unmethylated control DNAs (bisulphide modified); W control genomic DNA (not modified). A negative PCR control for each set with no DNA was used. A 100 base pair marker was used at the beginning of the gel electrophoresis. Visualization was done by UVP gel visualization system. DNA was considered as methylated if a specific PCR product using methylation specific primers was seen regardless of the signal obtained with the non-methylation specific primer [13].

Statistical Analysis

Kruskal Wallis nonparametric test was used to compare proportions between study groups. P value of less than 0.05 was considered statistically significant.

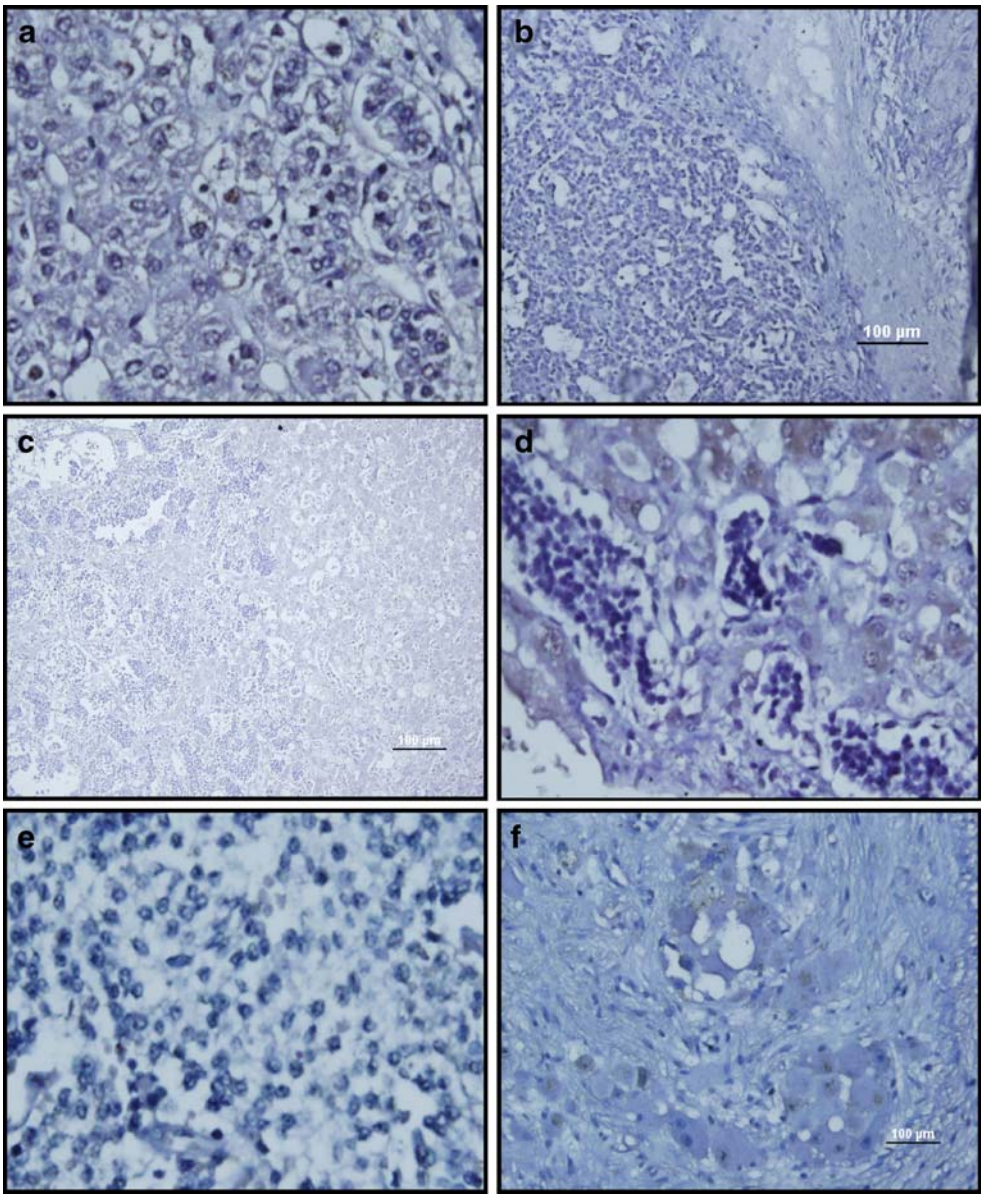

Fig. 1 P16 Expression in different case groups (DAB). **a** Adrenal medulla p16 (nuclear +), **b** Adrenal Blastic Rest p16 (−), **c** Neuroblastoma in situ p16 (−), **d** Neuroblastoma in situ at high power field, **e** Neuroblastoma p16 (−), **f** Differentiating neuroblastoma after chemotherapy p16 (nuclear +)

Results

Clinical and Pathological Features of the Series

This study included 23 cases (11 male; 12 female) and 31 samples from archival paraffin embedded tissues. The age range of autopsy cases was between 20 weeks of foetal age and 36 months of infant age. The mean age of NB cases was 45 months. The mean treatment time of the NB cases was 6 months. The cases were treated according to the Turkish NB Treatment Protocol, which is the same with SIOP protocol. All of the cases were stage III or IV. The cases were not resistant to chemotherapy decided according to remission results to induction therapy. All eight cases are alive in a median 3 years follow up. According to Modified Shimada System all of the NB cases were in unfavourable category.

Frequency of Expression

P16 expression was positive in normal adrenal tissue samples, in one of 5 samples of adrenal blastic rest tissue and in all of the samples of after chemotherapy; while no expression was observed in samples of NB and NB in situ tissues (Fig. 1) (Table 1). The expression was nuclear. The intensity was not taken into consideration.

Frequency of Methylation

P16 methylation was observed in samples of NB in situ and primary NB tissues (Fig. 2) (Table 2).

Statistical Analysis

In Kruskal-Wallis nonparametric test, p16 expression (p= 0.001) and p16 methylation (p=0.003) were found to be

Table 1 The properties and p16 expression and methylation status of the cases

No	Case	Age/sex	P16 Expression	P16 Methylation
1	Adrenal medulla	3 years/M	+	–
2	Adrenal medulla	2.5 months/M	+	–
3	Adrenal medulla	21 weeks featus/M	+	–
4	Adrenal medulla	15 days/F	+	–
5	Adrenal medulla	32 days/F	+	–
6	Adrenal blastic rest	31 weeks featus/F	–	–
7	Adrenal blastic rest	25 weeks featus/M	–	–
8	Adrenal blastic rest	20 weeks featus/F	+	–
9	Adrenal blastic rest	4/365 days/F	–	–
10	Adrenal blastic rest	2 months/M	–	–
11	Neuroblastoma in situ	21 week featus/M	–	+
12	Neuroblastoma in situ	21 week featus/M	–	+
13	Neuroblastoma in situ	21 week featus/M	–	+
14	Neuroblastoma in situ	21 week featus/M	–	+
15	Neuroblastoma in situ	7/365 day/F	–	+
16	Neuroblastoma, primary	12 months/M	–	+
	After chemotherapy		+	–
17	Neuroblastoma, primary	13 months/M	–	+
	After chemotherapy		+	–
18	Neuroblastoma, primary	30 months/F	–	–
	After chemotherapy		+	–
19	Neuroblastoma, primary	5.5 years/F	–	+
	After chemotherapy		+	–
20	Neuroblastoma, primary	3 years/F	–	+
	After chemotherapy		+	–
21	Neuroblastoma, primary	10 years/F	–	+
	After chemotherapy		+	–
22	Neuroblastoma, primary	3 years/F	–	–
	After chemotherapy		+	–
23	Neuroblastoma, primary	4 years/F	–	+
	After chemotherapy		+	–

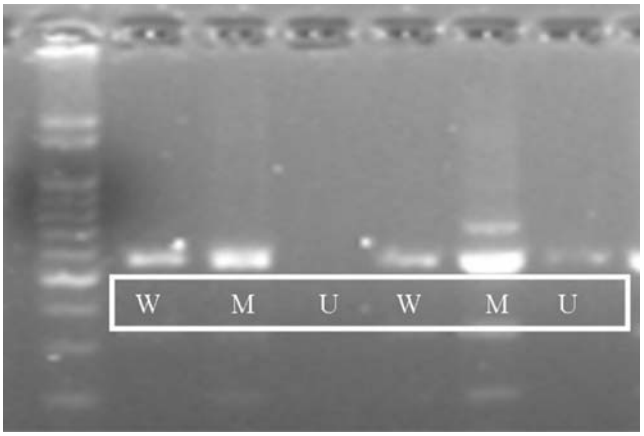

Fig. 2 P16 methylation shown by gel electrophoresis *W*: wild control, *M*: methylated, *U*: unmethylated

statistically significantly different in diagnostic groups. P16 expression was present in adrenal medullar tissue samples and in tumour tissue samples after multimodel chemotherapy treatment. In contrast, p16 methylation was observed in samples of NB in situ and primary tumour tissues.

Discussion

In this study, p16 expression and methylation were studied and compared in 5 samples of normal adrenal medullar tissue, 5 samples of adrenal tissue including blastic rests, 5 samples of NB in situ tissue and in 8 samples of NB tissue before and after chemotherapy.

A distinct adrenal medulla is not present in foetus, but it is almost formed at birth. In foetus, however chromaffin cells are haphazardly scattered throughout the foetal cortex. Neuroblastic cells from the neural crest migrate through the cortex, than they transform to adrenal medullar cells by maturation [11]. After 20 weeks of foetal life, blastic cells are not expected in adrenal medulla. Congenital NB is known to regress spontaneously. Adrenal medulla or migrating neuroblastic cells are thought to be precursors of NB. With this background the adrenal medullar cell spectrum that are blastic in an unexpected foetal or postnatal life time and in this unexpected morphology was remarkable for us to be studied in NB tumorigenesis model. For paediatric NB, foetal adrenal sympathetic neuroblasts are assumed to be the cells of origin, but these cells are virtually absent after birth and thus not readily available for analysis [14]. Our study was the first one to study methylation in adrenal neuroblasts.

Protein complexes of D-type cyclins and CDKs (CDK4 and CDK6) induce the phosphorylation of pRb to promote the G1-S-phase transition. The phosphorylated pRb releases transcriptional factors such as E2F, which activate the expression of genes essential for S-phase entry [15]. CDK inhibitor proteins, including p16, play critical roles in the G1-S cell cycle transition by inhibiting the cyclin D-CDK4/6-mediated pRb phosphorylation [16]. Omura-Minamisawa et al. interestingly, found that the samples in the unfavorable stages exhibited expression of p16 mRNA and protein more frequently than those in the favorable stages [4]. Bassi et al. studied mutations and deletions of p16 and p15 in 11 NB patients. They used multiplex PCR technique for homozygous deletion analysis and; single-strand conformation polymorphism and nucleotide sequencing for mutation analysis. By these methods they analyzed all exons of both genes, but no deletion was detected [17]. In previous studies hypermethylation of tumor-suppressor genes is found to be related to NB evolution. These epigenetic alterations could be used as a marker of the disease, and also as potential therapeutic targets. Michalowski et al. found RASSF1A to be hypermethylated in 100% of cell lines and in 50–90% of tumor specimens. Other genes that were frequently methylated in NBs were CASP8, DcR1, DcR2, genes mainly associated with apoptosis [2].

There has been an increased interest in methylation in last 10 years. The methyl group at the fifth position of the cytosine pyrimidine ring, that is present in about 80% of CpG-dinucleotides in the human genome, can be of major functional significance and is regarded as the ‘fifth base’ of the genome [18]. DNA methylation, along with histone modifications (acetylation, methylation, phosphorylation and etc), are referred to as epigenetic phenomena that control various genomic functions without a change in nucleotide sequence [19]. Regulation of developmental programming and cell differentiation is affected by methylation [20].

Holst et al. showed that epithelial cells with methylation of *p16* promoter sequences occur in focal patches of histological normal mammary tissue of a substantial fraction of healthy, cancer-free women. They concluded that this observation of *p16*-methylated variant cells in

Table 2 Comparison of p16 expression or methylation status according to case groups

	Adrenal medulla	Adrenal blastic rest	Neuroblastoma in situ	Neuroblastoma, primer	Neuroblastoma, After chemotherapy
p16 Expression (n)	5/5	1/5	0/5	0/8	8/8
p16 Methylation (n)	0/5	0/5	5/5	6/8	0/8

histological normal tissue might be identifying premalignant lesions before the morphological changes [13].

Nuovo et al. used MSP-ISH to monitor the timing and consequences of aberrant hypermethylation of the *p16* tumor suppressor gene during the progression of cancers of the lung and cervix and they suggested that p16 inactivation is selected as the most effective mechanism of blocking the cyclin D-Rb pathway during the evolution of an invasive cancer from precursor lesions. These studies demonstrate that MSP-ISH is a powerful approach for studying the dynamics of aberrant methylation of critical tumor suppressor genes during tumor evolution [20].

It was reported that inactivation of the p16 gene is involved in the progression of NB independently of N-myc amplification [3]. That is why we did not correlate our results with nmyc amplification of the tumors.

In conclusion; our results suggest that cyclin dependent kinase inhibitor p16 and its methylation seem to play roles in NB tumorigenesis and in the migration, population and differentiation of primordial neural crest cells. In this study, in normal adrenal medulla, p-16 protein is found to be expressed and not methylated; while, in neuroblastoma in situ and primer neuroblastoma cases, it is not expressed and methylated. Our results indicate a started carcinogenesis in the in situ lesions. Post chemotherapy re-expression of p16 might be a reactive postchemotherapy event due to the DNA damage caused by the cytostatic drugs and/or might be one of the mechanisms of drug effect. Inhibitors of DNA methylation may provide a useful tool for restoring p16 activity in NB treatment.

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