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IMMUNOHISTOCHEMICAL EXPRESSION OF PROTEIN p16 IN WILMS' TUMOR

BASTA-JOVANOVIĆ GORDANA*, SUZIĆ SLAVICA**, SAVIN MARINA*****, PETRONIJEVIĆ NATAŠA***, RADONJIĆ NEVENA***, ĐURIČIĆ S****, JOVANOVIĆ MILENA* and RADOJEVIĆ-ŠKODRIĆ SANJA*

**Institute of Pathology, School of Medicine, University of Belgrade, Serbia*
***Institute of phisiology, School of Medicine, University of Belgrade, Serbia*
****Institute of biochemistry, School of Medicine, University of Belgrade, Serbia*
*****Institute for mother and child health protection "Dr Vukan Čupic", Belgrade, Serbia*
******Institute for nephrology, Clinical Center of Serbia, Belgrade, Serbia*

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Proliferative disorders including tumors are recognized as diseases of the cell cycle in which the crucial role belongs to the cyclin-dependent kinase family. The aim of this study was to investigate the expression of protein p16 in the normal kidney, and in Wilms tumor by immunohistochemistry to correlate the obtained results with tumor stage, histological type and prognostic group. We have investigated 28 cases of Wilms tumor, two Wilms tumor metastases in the lungs and one case of normal renal tissue.

Protein p16 was over expressed in 20 cases of Wilms tumor. Expression of p16 was less frequent in stages III/IV/V than in stages I/II, showing no statistically significant correlation ($p>0.05$). Intermediate risk groups of Wilms tumor showed more frequent p16 expression in comparison with high risk cases, showing no statistical significance ($p>0.05$). Protein p16 expression was detected in all histologic types of Wilms tumor with the highest expression in the predominantly blastemal type (50%). There was a statistically significant difference between p16 expression in this and its expression in other histological types ($p<0.05$). The correlation between p16 expression and the predominantly blastemal histological type was statistically significant and showed that in such cases tumor cells can proliferate even in the presence of p16.

Key words: cell cycle, protein p16, immunohistochemical, swine, Wilms tumor

INTRODUCTION

Several pathways need to be abrogated in the genesis of a malignant neoplasm. Deregulation of one or more cell cycle checkpoints is among the most common abnormalities in human neoplasia. Molecules that play key roles in the control of cell-cycle progression are the cyclin-dependent kinases (CDKs, protein

complexes that are composed of a regulatory cyclin subunit and a catalytic partner), their substrate proteins and the CDK inhibitors (CDKIs). Cyclins form complexes with specific CDKs at distinct points in the cell cycle and phosphorylate target proteins and promote cell-cycle progression. By contrast CDKI molecules bind to, and inhibit the activities of specific cyclin-CDK complexes, thereby causing cell-cycle arrest (Brooks *et al.*, 1998; Li and Brooks, 1999).

Proliferative disorders such as cancer are recognized as diseases of the cell cycle. It has generally been found that in tumor cells, the mechanisms that normally function to restrain cell division are defective, while those that promote division become more active. Both positive (cyclins and CDKs) and negative (CDKIs) regulators of the cell cycle that function upstream of pRb (the product of the retinoblastoma gene), can be aberrantly controlled during proliferative diseases. It has been shown that cyclin D1, CDK4 and CDKI molecules ($p15^{INK4B}$ and $p16^{INK4A}$) are subject to mutational events in human tumor cells (Kamb *et al.*, 1994; Zwijsen *et al.*, 1998).

Protein p16, a member of INK4A family, is a cell cycle inhibitor, commonly inactivated in human tumors. Despite its importance in human neoplasia, the normal pattern of p16 expression remains largely unknown (Nielsen *et al.*,1999).

Nephroblastoma, a relatively common renal neoplasm of young pigs, represents the animal counterpart of Wilms tumor in children (Grieco *et al.*, 2006). Animal nephroblastoma has the same morphological features as Wilms tumor in children. It usually presents between the ages of 3 and 6 years in humans, as well as in animals (Matsunaga, 1981; Breslow and Langholtz, 1983). In humans, most Wilms tumors occur sporadically and are unilateral, but there are rare (1%) familiar cases, which are often bilateral and are diagnosed at an earlier age (Matsunaga, 1981; Breslow and Langholz, 1983; Fukuse T *et al.*, 2000). Wilms tumors occur most frequently in swines as sporadic tumors (Tsurutani *et al.*, 1998).

The aim of this study was to investigate the expression of protein p16 in the normal kidney, as well as in Wilms tumor by immunohistochemistry and to correlate the results with tumor stage, histological type and prognostic group.

MATERIAL AND METHODS

According to Declaration of Helsinki, tumor specimens used in this study were obtained from 28 patients undergoing surgery for Wilms' tumor at the Institute for Mother and Child Health Protection "Dr Vukan Čupić", Belgrade (F:M ratio 18:10; age 7-132 months). A total of 9 cases was not treated with preoperative chemotherapy, and 2 metastases of Wilms' tumor found in the lungs and one normal kidney specimen were also analyzed**.** Formalin-fixed and paraffin-embedded samples of all specimens were examined in the Institute of Pathology, School of Medicine, University of Belgrade.

SIOP (International Society of Paediatric Oncology) classification from 2002 was used to determine tumor stage, histological type and prognostic group of tumor (Vujanić *et al*., 2002). According to this classification, 17 (60.7%) out of 28 cases was classified as Wilms tumor stage I, 4 (60.7%) as stage II, 4 (14.3%) as

stage III and 2 (7.1%) as stage IV. One case (3.6%) of bilateral Wilms tumor was analyzed (stage V) and it was found that the tumor in the left kidney was stage I, while the tumor in the right kidney was stage II. Two (7.1%) out of 28 analyzed cases were predominantly of the epithelial type, 11 (39.3%) blastemal, 6 (21.4%) stromal, and 4 (14.3%) were typical mixed type. In five cases the tumor was composed of anaplastic cells, 4 (14.3%) cases were diffusly anaplastic, and 1 (3.6%) contained focal anaplasia. 18 (64.3%) cases were classified as intermediate prognostic group, and 10 (35.7%) as high risk group.

For immunohistochemistry, 5 μm-thick sections were cut from one block per patient and heated for 20 min in a microwave oven in a solution of 0.1 M citrate buffer (pH=6.0), three cycles for 5 minutes. Endogenous peroxidase activity was quenched by treating the slides with 3% H_2O_2 for five minutes. To block the nonspecific reaction normal swine serum (dilution 1:10) was applied for 30 minutes. Incubation with the primary polyclonal antibody against p16 (F-12, Santa Cruz Biotechnology, USA) was performed at a concentration 1:100 over night at room temperature. Streptavidin-biotin staining method was performed using DAKO LSAB+ kit. 3,3diaminobenzidine (DAB) was used as chromogen and for contrasting Mayers hematoxylin.

The results of immunohistochemical staining were scored by the semiquatitive technique: negative staining (-), positive staining involving 10% positive cells (focal expression; +), 10%-50% positive cells (moderate expression; ++), and more than 50% (diffuse expression; +++). Statistic evaluation was done by Fisher's test, Mann-Whitney's and Student's T- test. P value <0.05 was considered as statistically significant. Statistical comparison between the two tumor groups was done. One group was represented by cases without or with focal p16 expression (absence of expression). The second group was represented by cases with moderate and diffuse p16 expression (diffuse expression).

RESULTS

Clinical and morphological features and expression of protein p16 in Wilms tumor are presented in Table 1. Protein p16 expression was detected in epithelial cells of distal convoluted tubules, in interstitial cells, in glomerular parietal epithelial cells, as well as in podocytes and endothelial cells of glomerular capillaries in normal, unchanged human kidneys (Figure 1A). In our group of 28 Wilms tumor cases we have detected p16 expression in 20 cases (71.43%). Diffuse expression of protein p16 was more frequent in blastemal (75%;without epithelial component) then in epithelial (69.2%; without blastemal) component. This correlation did not show statistical significance ($p>0.05$). Expression of p16 was less frequent in stages III/IV/V (42.85%) than in stages I/II (80.95%), showing no significant correlation ($p>0.05$). Intermediate risk cases of Wilms tumor showed more frequent p16 expression (83.33%) in comparison with high risk cases (50%), showing no significant difference ($p>0.05$). Protein p16 expression was detected in all histologic types of Wilms tumor with the highest expression in the predominantly blastemal type (50%). There was a significant difference

Table 1. Clinical – morphological features and expression of protein p16 in Wilms

Number	Therapy	Age (months)	Genderl	Size of tumor (cm)	Stage	Histological type	Prognostic group	E	EE	EB
1	-	7	M	7	I	blastemal	IM	++	+	++
2	-	43	F	9	I	blastemal	IM	++	0	++
3	+	23	F	7	I	mixed	IM	++	++	++
4	+	14	F	8	II	stromal	IM	++	++	++
5	+	18	F	7	IV	stromal	IM	++	++	++
*										
6	+	24	F	7	I	stromal	IM	++	++	++
7	+	48	F	6	I	mixed	IM	++	+	++
8	+	36	F	6	I	blastemal	HR	+	+	+
9	+	60	F	6	I	blastemal	HR	++	++	++
10	+	24	M	8	III	focal anaplasia	IM	+	+	+
11	+	84	M	8	III	diffuse anaplasia	HR	+	+	+
12	+	132	M	12	I	blastemal	HR	++	++	++
13	+	36	F	7	II	blastemal	HR	++	++	++
14	+	84	M	13	I	blastemal	HR	++	++	++
15	+	24	F	10	I	epithelial	IM	+	+	+
16	+	12	F	10	I	epithelial	IM	++	++	++
17	+	96	F	16	III	stromal	IM	++	++	++
18	+	132	F	19	III	diffuse anaplasia	HR	+	++	++
19	+	24	F	6	I	stromal	IM	++	++	++
20	+	57	M	8	II	blastemal	HR	++	++	++

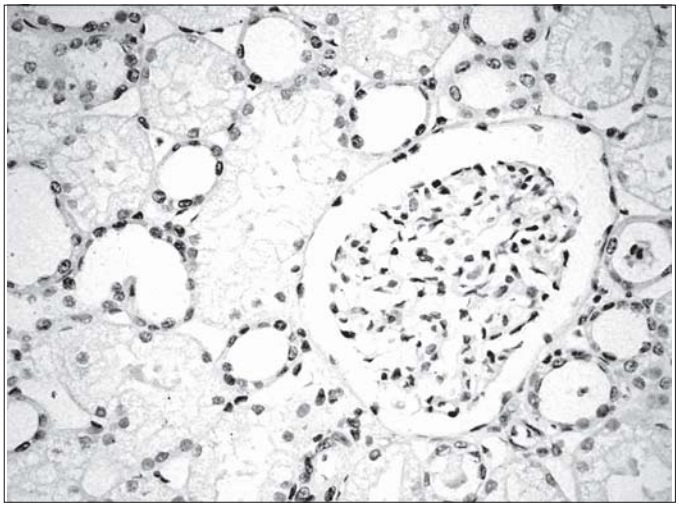
Cont. Table 1.

Number	Therapy	Age (months)	Genderl	Size of tumor (cm)	Stage	Histological type	Prognostic group	E	EE	EB
21	-	55	M	8	I	diffuse anaplasia	HR	+	+	+
22	-	25	F	7	IV	blastemal	IM	++	++	++
*										
23	-	64	F	3	I	blastemal	IM	++	++	++
24	-	52	F	4	V	stromal	IM	+	+	+
25	-	16	F	5	I	mixed	IM	++	++	++
26	-	79	M	9	I	blastemal	IM	++	++	++
27	-	55	M	16	I	diffuse anaplasia	HR	+	0	+
28	+	79	M	5	II	mixed	IM	++	++	++

Legend:
M – male; F – female; E – p16 expression (total); EE – p16 expression only in the epithelial component;
EB – p16 expression only in the blastemal component; HR – high risk group; IM – intermediate prognostic group;
"*" – metastatic of Wilms tumour in lung tissue
"0" – there is no epithelial component in Wilms tumour specement

between p16 expression in this and expression in other histological types ($p<0.05$). Two cases of metastases in lungs showed diffuse p16 expression (Figure 1B), but one case of bilateral Wilms tumor showed no p16 expression.

A



B

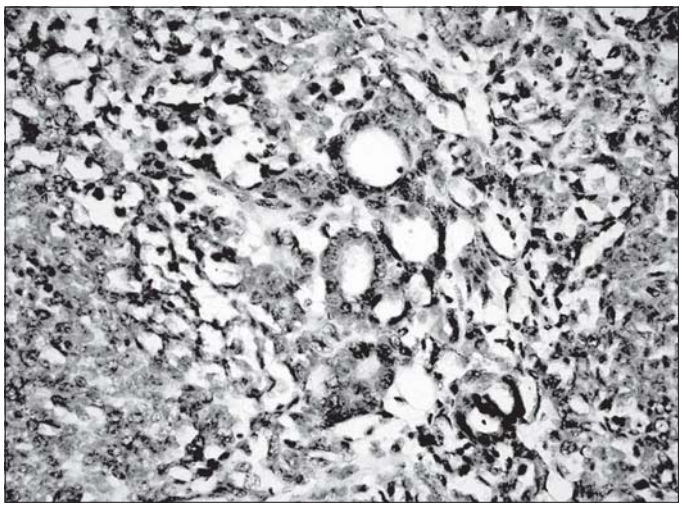


Figure 1. A. Diffuse expression of protein p16 in normal kidney tissue.
B. Diffuse and pronounced expression of protein p16 in metastasis of Wilms tumor. Streptavidin-Biotin, x400

DISCUSSION

Factors that determine whether the cell will continue to proliferate, or stop it's growth and differentiation, express their activities during G_1 cell cycle phase. The beginning of the S cell cycle phase depends on the coordinated activity of a small serine/threonine kinase familly (CDK- cyclin dependent kinase) (Brooks *et al.*,1998). CDK play the key role in G_1 phase. Regulation of CDK function is crucial both for proliferative activity of the cell, and for its differentiation. Cyclins activate CDK, while CDKI protein group (cyclin dependent kinase inhibitor) acts in the opposite direction. Two inhibitor groups are identified: INK4 and KIP/CIP proteins. Abnormal regulation of cell cycle can result in uncontrolled cell growth and cancer formation. Cyclin's over expression results in rapid cell proliferation and cancer formation (Zwijsen *et al.*, 1998). Therefore, immunohistochemical detection and evaluation of expression of cyclins and their inhibitors can be used for tumor growth follow up. We presumed it would be of significance to analyze the expression of cell cycle regulators in Wilms tumor as markers of cell cycle proliferation and differentiation, that could have an impact on effective therapy. In our previous study we showed that proteins, regulators of apoptosis, detected by immunohistochemistry play an important role in the development and biological behaviour of Wilms tumor (Basta-Jovanović *et al.*, 2005). Literature data reports of immunohistochemical investigations of cell cycle regulators in Wilms tumor (Nagoshi and Tsuneyoshi, 1994; Arcellana-Panlilio *et al.*, 2000; Ghanem *et al*., 2001; Radojević-Skodrić *et al.*, 2007), but our study is the first one to examine protein p16 expression by immunohistochemistry in Wilms tumor (Radojević S, 2002).

In normal renal tissue we have found protein p16 nuclear expression in interstitial cells, epithelial cells of distal convoluted tubules, parietal epithelial cells, podocytes and in endothelial cells of glomerular capillary loops. In our group of 28 Wilms tumor cases we have detected p16 expression in 20 cases (71.43%). Expression of p16 was less frequent in stage III/IV/V (42.85%) than in stage I/II (80.95%), contratry to literature data (Arcellana-Panlilio *et al.*, 2000; Kawabuchi B *et al.*, 1999; Zhang *et al.*, 2002). We have found a significant correlation between the p16 expression and the predominantly blastemal histological type ($p<0.05$). p16 expression was more frequent in Wilms tumor cases in the intermediate group then in high risk cases, but with no statistical significance ($p>0.05$). Similar findings were published by Takite and coworkers in neuroblastoma (Takita *et al*., 1998).

In our investigation we have examined two cases of Wilms tumor metastases in the lungs. In both cases we have found a diffuse p16 expression, opposite to the published findings of Straume and coworkers who found a decreased p16 expression in melanoma metastases in comparison with the primary melanoma (Straume *et al.*, 2000). Geradts and Ingram, 2000 showed that the abnormal p16 expression was closely connected to tumor size. We did not have the same experience in our study ($p>0.05$).

CONCLUSION

Correlation between p16 expression and the predominantly blastemal histlogical type was statistically significant ($p<0.05$) and showed that in these cases tumor cells can proliferate even in the presence of p16.

Address for correspondence:
Gordana Basta-Jovanović
Jaše Prodanovića 32,
11000 Belgrade
Serbia
E-mail: jovanovic01@yahoo.com

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Basta-Jovanović Gordana *et al.*: Immunohistochemical expression of protein p16 in Wilms' tumor

IMUNOHISTOHEMIJSKA EKSPRESIJA PROTEINA p16 U WILMS-ovom TUMORU

BASTA-JOVANOVIĆ GORDANA, SUZIĆ SLAVICA, SAVIN MARINA, PETRONIJEVIĆ NATAŠA, RADONJIĆ NEVENA, ĐURIČIĆ S, JOVANOVIĆ MILENA i RADOJEVIĆ-ŠKODRIĆ SANJA

SADRŽAJ

Proliferativne bolesti, uključujući tumore, predstavljaju poremećaje regulatora ćelijskog ciklusa koji pripadaju familiji ciklin-zavisnih kinaza. Cilj ovog rada je bio da se analizira ekspresija proteina p16 u normalnom tkivu bubrega kao i u Wilms-ovom tumoru i da se ispita odnos te ekspresije sa histološkim tipom, stadijumom i prognostičkom grupom tumora. Ukupno je analizirano 28 slučajeva Wilms-ovog tumora, dve metastaze i jedan uzorak normalnog tkiva bubrega. Difuzna ekspresija ciklina A je uočena mnogo češće u blastemskoj nego u epitelnoj komponenti (50%:34,6%) i ova korelacija je bila statistički značajna ($p<0,05$). Difuzna ekspresija ciklina A je mnogo češća u višim stadijumima (III/IV/V; 85,7%) nego u nižim (I/II; 33,24%), uz statističku značajnost na nivou $p<0,05$. Slučajevi Wilms-ovog tumora koji pripadaju grupi srednjeg stepena rizika, imali su ćećšu ekspresiju ciklna AA (55,6%) u odnosu na slučajeve iz visoko rizične grupe (30%), ali bez statistički značajne korelacije ($p>0,05$). Difuzna ekspresija proteina p16 je uočena u 20 slučajeva Wilms-ovog tumora. Protein p16 je znatno ređe eksprimiran u slučajevima stadijuma III/IV/V u odnosu na stadijume I/II, ali ova korelacija nije bila statistički značajna ($p>0,05$). Slučajevi iz srednje grupe rizika su češće imali eksprimiran protein p16 u poređenju sa slučajevima iz visoke grupe rizika, ali bez statistički značajne korelacije ($p>0,05$). Difuzna ekspresija p16 je mnogo češća u predominantno blastemskom tipu tumora (50%) u odnosu na ostale histološke tipove i ova korelacija je bila statistički značajna ($p<0,05$). Korelacija između difuzne ekspresije proteina p16 i predominantno blastemskog histološkog tipa ukazuje da tumorske ćelije proliferišu i u prisusutvu proteina p16.