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IMMUNOHISTOCHEMICAL ANALYSIS OF CYCLIN E IN WILMS TUMORS

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Abnormalities in cell cycle regulators and subsequent deregulation of G1-S transition may be one of the most important biological events in the malignant transformation of cells. The aim of this study was to investigate the expression of Cyclin E in the normal kidney, and in Wilms tumor, and to correlate the results with tumor stage, histological type and prognostic group.

We have investigated 28 cases of Wilms tumor, two Wilms tumor metastases in lungs and one case of normal renal tissue. Correlation of semiquantitatively scored cyclin E levels with histopathological parameters was performed for all cases.

Diffuse expression of cyclin E was more frequent in the blastemal (without epithelial component) then in the epithelial (without blastemal) component. This correlation showed high significance ($p=0.001$). Diffuse expression of cyclin E was more frequent in stage III/IV/V (71.4%) than in stage I/II (42.9%), showing no significant correlation ($p>0.05$). The intermediate risk group of Wilms tumor showed more frequent cyclin E diffuse expression (66.7%) in comparison with high risk cases (20%), showing statistical significance ($p<0.05$).

Our findings suggest that diffuse expression of cyclin E is associated with the prognostic group and tumor stage. Also, cyclin E expression is more pronounced in the blastemal component than in the epithelial component.

Key words: cyclin E, cell cycle, swine, histological type, prognostic group, tumor stage

INTRODUCTION

Cyclins are prime cell cycle regulators and play a central role in the control of cell proliferation by forming complexes with different cyclin-dependent kinases (CDKs) (Sherr, 1993). These complexes enzymatically phosphorylate cell cycle regulatory elements, such as the retinoblastoma protein (Rb). Cyclin D1 and E are

responsible for the activation of CDKs during the G1 phase and they are rate-limiting factors for G1-S transition (Resnitzky and Reed, 1995).

Cyclin E is a highly conserved protein that was first identified by virtue of its ability to rescue G1 cyclin-defective budding yeast (Koff *et al*., 1991) which, along with the catalytic subunit CDK2, is involved in the phosphorylation of the retinoblastoma protein. Increased expression of cyclin E has been shown to reduce the length of G1 phase and accelerate the transition into S-phase (Ohtsubo *et al.*, 1995).

Abnormalities in cell cycle regulators and subsequent deregulation of G1-S transition may be one of the most important biological events in the malignant transformation of cells. Cyclin E over-expression has been found in several cancers (Keyomarsi *et al*., 1994; Dutta *et al*., 1995; Nielsen *et al.,* 1996; Wang *et al*., 1996; Sakaguchi *et al.*,1998; Mishina et al., 2000; Fukuse *et al*., 2000).

Nephroblastoma, a relatively common renal neoplasm of young pigs, represents the animal counterpart of Wilms tumor of children (Grieco *et al.*, 2006). Animal nephroblastoma has the same morphological features as Wilms tumor in children. It shows the three components typical of Wilms tumor: mesenchymal blastema, epithelium (tubuli and glomeruloid bodies) and stroma (Grieco *et al.*, 2006). It usually presents between the ages of 3 and 6 years in humans, as well as in animals (Matsunaga, 1981; Breslow and Langholtz, 1983). It is highly responsive to chemotherapy. Affected children usually have a good prognosis with a reported 5-year survival rate in more than 80% of cases (Exelby, 1991). In humans, most Wilms tumors occur sporadically and are unilateral, but there are rare (1%) familiar cases, which are bilateral and are diagnosed at an earlier age (Matsunaga, 1981; Breslow and Langholz, 1983; Fukuse *et al.*, 2000). Wilms tumors occur most frequently in swines as sporadic tumors (Tsurutani *et al.*, 1998).

The molecular events involved in Wilms tumor development are still poorly understood. Loss of heterozygosity studies have implicated several distinct tumor suppressor genes in the development of Wilms tumors (Koesters *et al.*, 2002).The nucleotide sequence and expression patterns in organs of pWT1 (porcine WT1 gene) were similar to those of human WT1 (Tsurutani *et al.*, 1998).

The aim of this study was to investigate the expression of cyclin E protein in 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 Helsinki Declaration, tumor specimens used in this study were obtained from 28 patients undergoing surgery for Wilms' tumor at University Children Hospital, Belgrade and at Institute for Mother and Child Health Protection "Dr Vukan Cupic", Belgrade (F:M ratio 18:10; age 7-132 months). A total of 9 cases was not treated with preoperative chemotherapy. Two Wilms' tumor metastases 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.

To determine tumor stage, histological type and prognostic group of tumor SIOP (International Society of Paediatric Oncology) classification from 2002 was used (Vujanic *et al.*, 2002). According to this classification, 17 (60.7%) out of 28 cases were 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%) with 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 epithelial type, 11 (39.3%) blastemal, 6 (21.4%) stromal, and 4 (14.3%) were tipical mixed type. In five cases the tumor was composed of anaplastic cells, 4 (14.3%) and 1 (3.6%) contained focal anaplasia. 18 (64.3%) cases were classified as intermediate prognostic group, and 10 (35.7%) were in the high risk group.

For immunohistochemistry, 5 μm-thick sections were cut from one block per case 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 cyclin E (M-20, Santa Cruz Biotechnology, USA) was performed at a concentration 1:600 overnight 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 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 cyclin E expression only (absence of expression). The second group was represented by the cases with moderate and diffuse cyclin E expression (diffuse expression).

RESULTS

Normal kidney tissue did not express cyclin E (Figure 1).

In the group of 28 Wilms tumor cases we have detected diffuse cyclin E expression in 14 cases (50%). Clinical-morphological features and expression of cyclin E in Wilms are presented in Table 1.

Diffuse expression of cyclin E was more frequent in the blastemal (without epithelial component) then in the epithelial (without blastemal) component (50%:28%). This relation showed a high statistical significance ($p=0.001$). Diffuse expression of cyclin E was more frequent in stage III/IV/V (71.4%) than in stage I/II (42.9%), showing no significant correlation ($p>0.05$). There was no significant correlation between cyclin E diffuse expression in blastemal (without epithelial component; $p>0.05$), or in epithelial component (without blastemal component; $p>0.05$) and tumor stage.

Table 1. Clinical - morphological features and expression of cyclin E in Wilms

Number	Therapy	Age (monthsi)	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	-	0	+
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 (monthsi)	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	++	++	++

Legends:
M – male
F – female
E – cyclin e expression in all component (total)
EE – cyclin e expression only in epithelial component
EB – cyclin e expression only in blastemal component
"0" – there is no component in Wilms tumor
HR – high risk group
IM – intermediate prognostic group
"*" – metastatic of Wilms tumor in lung tissue

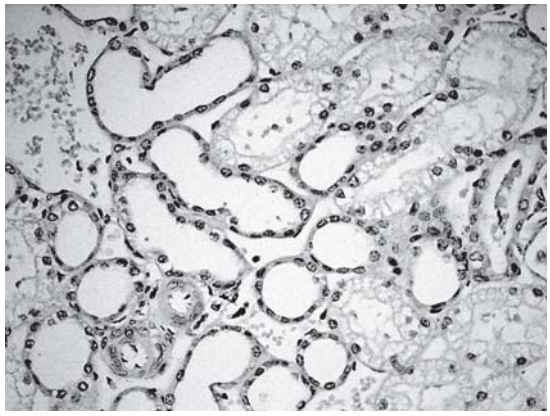

Figure 1. Negative staining of Cyclin E in normal renal tissue. B. Streptaidin-Biotin, x400

Intermediate risk group of Wilms tumor showed more frequent cyclin E diffuse expression (66.7%) in comparison with high risk cases (20%), showing statistical significance ($p<0.05$). A correlation between blastemal expression (without epithelial expression) and prognostic group ($p<0.05$) was recorded. Cyclin E diffuse expression was found more often in the blastemal component of intermediate group tumors (66.7%) then in the blastemal component of high risk tumors (20%). There was not a statistically significant correlation ($p>0.05$) between epithelial diffuse expression (without expression in blastemal component) and prognostic group.

Diffuse cyclin E expression was detected in all histologic types of Wilms tumor, but without statistical significance ($p>0.05$). From four cases of diffuse anaplasia, one case showed diffuse cyclin E expression (Figure 2), but there was no significant difference between this and other histological types ($p>0.05$).

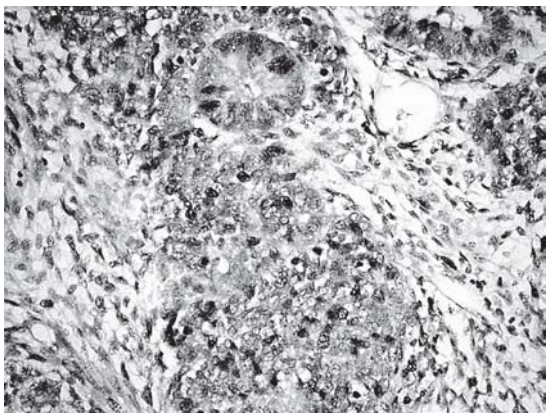

Figure 2. Diffuse expression of Cyclin E in anaplastic Wilms tumour. Streptavidin-Biotin, x400

Cyclin E immunostaining was not observed in two cases of metastasis nor in one case of bilateral Wilms tumor.

Our intention was to compare cyclin E diffuse expression in treated and untreated Wilms tumor patients. In treated patients diffuse cyclin E expression was more prominant in comparison with untreated cases (52.6% : 44.4%), but without a statistical significance ($p>0.05$).

DISCUSSION

Our study is the first one that examines cyclin E expression both in normal kidneys and in Wilms tumor. We have observed that normal kidney tissue does not show cyclin E expression. This finding confirms the well known fact that normal renal tissue has a low cell proliferative ability (Clapp and Cloker, 1997). Cell cycle disregulation can induce cancer formation. Immunohistochemical determination of the expression of various cyclins and CDKs in tumor cells has recently been applied to evaluate cancer growth (Bodey *et al.*, 1994; Dirks and Rutka, 1997).

Up to date, there are only few reports showing the results of immunohistochemical investigations in Wilms tumor (Re *et al.*,1999; Tanaka *et al.*,1999; Ghanem *et al*., 2001; Wunsch *et al*., 2001; Basta-Jovanovic *et al*., 2005). In literature, we have found only one report about PCR investigations of p16 cell cycle regulator in Wilms tumor (Arcellana-Panlilio *et al.*, 2000). Until now only few antibodies – vimentin, cytoceratins, smooth-muscle actin, Factor VIII and laminin were examined immunohistochemically in animal nephroblastomas (Grieco et al., 2006). According to our knowledge immunohistochemical analisys of cell cycle regulators in Wilms tumor was not performed until now.

We have examined cyclin E expression in normal renal tissue, in 28 cases of Wilms tumor and in two cases of Wilms tumor metastases in lungs. Literature data about cyclin E expression in different tumors are contraversial. According to some investigators, cyclin E is a unfavorable prognostic marker as diffuse expression is connected with poor prognosis (Yue H *et al*., 2003; Milde-Langosch *et al*., 2003). Other investigators showed opposite results (Kamai *et al*., 2001).

In 50% of Wilms tumor cases we have found cyclin E expression which was significantly more prominant in the blastemal than in the epithelial component (50%:28%; $p=0.001$). Higher cyclin E expression in the blastemal component was expected, because this component is less differentiated and has a higher proliferative activity.

Expression of cyclin E was more frequent in stages III/IV and V (71.4%) than in stages I and II (42.9%), showing no significant correlation ($p>0.05$). The relationship between cyclin E expression and tumor stage was found in other tumors, too (Scuderi *et al*., 1996; Sakaguchi *et al.*, 1998; Hedberg et al., 2002; Bedrosian *et al.*, 2004; Peters *et al.*, 2004; Rosen *et al*., 2006). However, in literature data we have also found opposite findings i.e. inverse relationship between cyclin E expression and tumor stage (Ito *et al.*, 1996; Muller-Tidow *et al*., 2001; Li *et al.*, 2001; Kamai *et al.*, 2001; Yue *et al.*, 2003; Milde-Langosch *et al*., 2003). It is well known that staging in Wilms tumor is accepted as a prognostic marker (the higher the stage, the worse the prognoses). Therefore, we have

assumed that diffuse cyclin E expression, detected in Wilms tumor, can indicate an unfavorable outcome.

We have found a significant correlation between diffuse cyclin E expression and prognostic group ($p<0.05$). We have noticed that intermediate risk tumors showed more prominent cyclin E expression in comparison to the high risk group (66.7%: 20% respectively). These results are opposite to our previous finding that cyclin E expression can indicate an unfavorable outcome in Wilms tumor. The possible explanation for this discrepancy in our results is that in SIOP classification, tumors are classified into prognostic groups according to histological types, regardless of tumor stage.

The expression of cyclin E was positive and increased in Wilms tumor in comparison to normal kidney tissue. On the other hand, in both cases of Wilms tumor metastases expression was negative, while the primary Wilms tumor, that is the origine of metastases, showed diffuse cyclin E expression. Due to a small number of cases, we can not conclude whether the diffuse expression of cyclin E can influence the ability of Wilms tumor to spread in the form of metastases. Such connection was demonstrated in cases of laryngeal squamous cell carcinomas and their metastases (Dong *et al*., 2000).

Our finding that cyclin E expression did not correlate to histologic type of Wilms tumor corresponded to literarure data, denying the connection of cyclin E expression and histologic type of bladder carcinoma (Makiyama *et al.*, 2000), as well as squamous cell carcinoma of lung (Datta *et al.*, 2000).

The anaplastic type of Wilms tumor is rare, and in comparison to the classical, tricomponent type, it gives metastases more often, is resistant to therapy, and it has a very bad prognosis. In our material we had only five cases of anaplastic variations of Wilms tumor.

According to SIOP classification, two cases of diffuse anaplasia, were classified as stage I, and two cases as stage III. Only in one case of diffuse anaplasia, that was classified as stage III, we have found a diffuse cyclin E expression. We think that it is necessary to investigate more cases of different stages of Wilms tumor with diffuse anaplastic changes to be able to come to any conclusion about cyclin E expression and presence of anaplasia in Wilms tumor.

One of the most important principles in chemotherapy is the fact that cancer cells in different phases of the cell cycle have different sensitivities to treatment procedures. In this respect, it is essential to understand all the phases from the beginning of the S phase, as interference with DNA replication is one of the most important principles of chemotherapy (Datta *et al.*, 2000). By determining different cyclins, the fractions of cells in these phases can be described. Cyclin E determines the fraction of cells in the G1 phase (McLaughlin, 1994). The increased cyclin E expression confirms that tumor cells are in the G1 phase, and as such, they are more sensitive to chemotherapy. We observed more prominent cyclin E expression in treated patients in comparison with untreated cases (52.6%:44.4%), but without a statistical significance ($p>0.05$). Unfortunately, we could not show that increased cyclin E expression is in correlation with a good tumor response to chemotherapy because all cases received preoperative chemotherapy according to different SIOPs protocols, before taking the samples.

In the future study, we are going to examine cyclin E expression in tumor tissue before and after receiving preoperative chemotherapy.

CONCLUSION

Our findings suggest that diffuse expression of cyclin E is associated with tumor stage and prognostic group. Also, cyclin E expression is more pronounced in the blastemal component than in the epithelial component. Diffuse expression of cyclin E is associated with an unfavorable outcome of Wilms tumor.

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IMUNOHISTOHEMIJSKA EKSPRESIJA CIKLINA E U WILMS-OVOM TUMORU

RADOJEVIĆ-ŠKODRIĆ SANJA, ĐURIČIĆ S, JOVANOVIĆ MILENA, DIMITRIJEVIĆ I, LAZIĆ M, STOJŠIĆ ZORICA, BRAŠANAC C i BASTA-JOVANOVIĆ GORDANA

SADRŽAJ

Poremećaj ekspresije regulatora ćelijskog ciklusa i posledična deregulacija G1-S prelaza može biti jedan od najvažnijih bioloških događaja u malignoj transformaciji ćelija. Povećana ekspresija ciklina E je dokazana u nekoliko tipova malignih tumora.

Analizirano je 28 slučajeva Wilms-ovog tumora, dve metastaze i jedan uzorak normalnog tkiva bubrega. Bojenje je vršeno streptavidin-biotin tehnikom, a ekspresija proteina određivana je semikvantitativno. Ciklin E je bio eksprimiran u 14 slučajeva Wilms-ovog tumora (50%). Ekspresija ciklina E je češća u blastemskoj nego u epitelnoj komponenti i ova korelacija pokazuje statističku značajnost ($p=0.001$). Između ekspresije ciklina E i stadijuma tumora nije bilo statistički značajne korelacije, iako je češće uočena ekspresija ciklina E u stadijumima III/IV/V u odnosu na I/II. Ekspresija ciklina E je češća u slučajevima iz grupe umerenog stepena rizika nego u slučajevima visokog stepena rizika i ova korelacija pokazuje statističku značajnost ($p<0.05$).Tretirani Wilms-ovi tumori su češće pokazivali ekspresiju ciklina E u odnosu na netretirane tumore, ali bez statističke

značajnosti ($p>0.05$). Ekspresija ciklina E je bila uočena u različitim histološkim tipovima Wilms-ovog tumora ali bez statističke značajnosti ($p>0.05$). Dva slučaja metastaza Wilms-ovog tumora i jedan slučaj bilateralnog tumora nisu imali eksprimiran ciklin E.