EXPRESSION OF MULTIDRUG RESISTANCE-RELATED PROTEIN (MRP-1), LUNG RESISTANCE-RELATED PROTEIN (LRP) AND TOPOISOMERASE-II (TOPO-II) IN WILMS' TUMOR: IMMUNOHISTOCHEMICAL STUDY USING TMA METHODOLOGY

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Key words: MRP (multidrug resistance related protein)/LRP (lung resistance protein)/TOPO-II (topoisomerase II)/Nephroblastoma/Xenograft/TMA-tissue microarray

Aims: MRP-1, LRP and TOPO-II are all associated with protection of the cells from the adverse effects of various chemotherapeutics. The aim of this study was to measure the expression of these proteins in Wilms' tumor (WT).

Materials and Methods: TMA block was constructed from 14 samples of WT's and from xenografts derived from them. Sections of the TMA were used for immunostaining against MRP-1, LRP and TOPO-IIa.

Results: All normal kidneys expressed MRP-1 but were either weakly or negatively stained for LRP and TOPO-IIa. In WT samples, MRP-1 was universally expressed, exclusively in the tubular component, while there was no expression of LRP and TOPO-IIa showed heterogeneous distribution. The xenografts varied in their MRP-1 and TOPO-IIa expression and exhibited weak/negative staining of LRP.

Conclusions: This study shows that although all the proteins evaluated, had different expression patterns in the tumor samples, the most prominent changes in expression were found for MRP-1. The exact clinical implications of these changes in expression and their relevance to the resistance of these tumors to chemotherapy requires further investigation. The finding of different expression profiles for the multidrug resistance proteins in the original WT's and their xenografts suggests that the results of animal cancer models may be difficult to interpret.

INTRODUCTION

The clinical outcome of chemotherapy for Wilms' tumor has markedly improved over the last two decades but unsuccessful treatment results are sometimes encountered especially in the case of recurrent disease. A key factor in such frustrating chemotherapeutic failures is generally considered to be intrinsic or acquired drug resistance and in order to improve the chemotherapeutic effects, it is essential to identify the mechanisms of such drug resistance and develop means for overcoming it.

To date, a large number of mechanisms for acquired multidrug resistance involving proteins responsible for protection of cells and tissues against a number of anti-cancer agents have been described. The multidrug resistance is associated with an ATP-dependent decrease in the cellular drug accumulation which can be attributed to over-expression of various ATP-binding cassette transporter proteins such as the multidrug resistance protein MRP-1 (ref.¹¹). MRP-1 is normally expressed in tissues that have either absorptive or eliminative roles (e.g. gut, liver, kidney) and has been found to be over-expressed at baseline in chemotherapy-resistant tumors (such as kidney and colon cancers) and also found to be up-regulated during disease progression following chemotherapy

in originally chemotherapy-sensitive tumors such as leukemia^{11, 12}. Lung resistance protein (LRP), like MRP-1, also contributes to drug resistance in various malignancies, and has been found to be expressed in the normal kidney, adrenal, heart, lung, muscle, thyroid, prostate, bone marrow and testis¹⁴. LRP has cross-membrane trafficking functions, hypothetically regulating the transport of a broad spectrum of substances and it is thought, carboplatin and cisplatin as well⁸. Unlike the MRP protein family and LRP, Topoisomerase-II is a nuclear enzyme. It affects multidrug resistance by altering the topologic state of the DNA thus facilitating DNA replication and indirectly circumventing the cytotoxic effects of various chemotherapeutic drugs⁷.

The aim of this study was to measure MDR-related protein expression in a series of samples from several different WT cases to assess their potential contribution to drug resistance.

MATERIALS AND METHODS

Briefly, up to four separate human tumor xenografts were established in 4-to-6-week-old male NOD-SCID mice (Taconic Farms, Germantown, NJ) by subcutaneous in-

Table 1. Expression of drug resistance proteins in WT.

Stain	MRP			TOPO II α			LRP		
Tissue	No stain	Weakly	Moderate to strongly positive	No stain	Weakly	Moderate to strongly positive	No stain	Weakly	Moderate to strongly positive
Norm kidney (intern control)	0	0	8/8 (100 %)	6/8 (75 %)	2/8 (25 %)	0	3/8 (37.5 %)	5/8 (62.5 %)	0
Human WT (tubular)	1/14 (7 %)	7/14 (50 %)	6/14 (43 %)	9/14 (64 %)	2/14 (14,5 %)	3/14 (21,5 %)	14/14 (100 %)	0	0
Human WT (blastema)	11/14 (78,5 %)	3/14 (21.5 %)	0	10/14 (71.5 %)	4/14 (28.5 %)	0	14/14 (100 %)	0	0
Xenograft	2/4 (50 %)	0	2/4 (50 %)	1/4 (25 %)	0	3/4 (75 %)	2/4 (50 %)	2/4 (50 %)	0

jection of 5×10^6 cells of each tumor type suspended in Matrigel into the thighs or shoulders.

TMA paraffin-embedded block was constructed from normal renal tissue, 14 samples of WT's from different patients, and from xenografts derived from them, according to our previously described techniques (Pinthus et al.). Each tumor sample was presented in the block by several cores, 0.6 mm in diameter. 4 Dm thickness sections from this TMA block were cut using a microtome mounted on a silane-coated microscope slide, stained with hematoxylin & eosin (H&E) and immunostained with antibodies against MRP-1, LRP and anti-TOPO-II. The antibodies were used in the following dilutions: rabbit anti-MRP-I polyclonal antibody (Alexis corporation USA) at 1:50, mouse monoclonal anti-LRP clone MRPM5 (Dako cytomation Copenhagen) at 1:20, and mouse anti-TOPO-IIa monoclonal antibody clone T3D1 (Dako cytomation Copenhagen), at 1:50. Antigen retrieval was performed at 650 W for 30 minutes in sodium citrate buffer pH 6.0. Nonspecific binding was blocked with 5% swine or rabbit serum in phosphate buffer solution (PBS). Sections were incubated with the primary antibodies at the given working dilutions overnight at 4 °C. As secondary antibodies, biotinylated anti mouse or rabbit immunoglobulins (Dako, Copenhagen, Denmark, 1 : 50) were applied for 30 minutes at room temperature. Detection was performed using the Strept ABCComplex/HRP kit (Dako, Copenhagen, Denmark). Diaminobenzidine substrate was used as a chromogen, according to the manufacturer's instructions. Sections were counterstained with hematoxylin. Staining was evaluated independently by two pathologists (E.F., J.S.). The degree of staining was assessed in comparison to bronchial epithelium staining, which stained positively for all the three markers investigated, and was graded semi-quantitatively according to the percentage of stained cells and their staining intensity. The samples were classified as either showing no stain, weak stain or as exhibiting moderate to strongly positive stain.

RESULTS

As mentioned before each TMA paraffin-embedded block was constructed from normal renal tissue, 14 samples of WT's from different patients, and from xenografts derived from them, according to our previously described techniques (Pinthus et al.). Each tumor sample was presented in the block by several cores, 0.6 mm in diameter. 4 Dm thickness sections from this TMA block were cut using a microtome mounted on a silane-coated microscope slide, stained with hematoxylin & eosin (H&E) and immunostained with antibodies E. Fridman, J. Skarda, J. H Pinthus, J. Ramon, Y. Mor s against MRP-1, LRP and anti-TOPO-II. 20 % of cores have been lost during the staining procedure. As already mentioned above the evaluation of immunohistochemical staining was done semiquantitatively was done at 200x magnification in five fields.

The summary of the data obtained and representative figures showing the characteristic immunostainings of the 3 studied markers in various tissues are presented in Figure 1 and in Table 1. All the normal kidney tissue samples expressed MRP-1 and were either weakly or negatively stained for LRP and TOPO-IIa. All samples of WT's were stained for MRP-1 (mainly in the tubular component of the tumor, but to a lesser extent than observed in the normal tissues and blastemal component of the Wilms tumour) No expression of LRP was detected in any histological subtype of Wilms tumour. Heterogeneous distribution of TOPO-IIa was observed in the WT samples (both in the tubular 2/14 (14.5 %) and in the blastemal components 4/14 (28.5 %)). The xenografts showed different pattern of expression of these proteins compared to the original WT tumors. They varied in their MRP-1 and TOPO-IIa expression level but unlike the original tumors, 50 % exhibited weak staining of LRP.

Fig. 1. Expression of drug resistance proteins in WT.

DISCUSSION

A considerable number of renal malignancies, including renal cell carcinomas (RCC's) and urothelial tumors of the upper collecting system, are unresponsive to chemotherapy. However, WT is chemo-responsive in the vast majority of cases, though there are still patients who present primarily with decreased responsiveness (intrinsic, primary drug resistance) or subsequently develop secondary resistance following the initial chemotherapeutic courses (acquired, secondary drug resistance). These differences in response might be attributed to different cellular mechanisms that mediate the intrinsic cellular drug resistance. The proteins associated with multidrug resistance are key players in this process and alteration in their expression level is considered to be of prognostic relevance in terms of the chemotherapeutic treatment outcome⁵.

Studies performed on normal renal tissue samples revealed that the various proteins associated with multidrug resistance, including LRP (ref.⁸), MRP and TOPO-II, are expressed⁷. In renal tumors, reports on the expression level of these genes are somewhat inconsistent. In RCC, Volm et al.¹⁸ reported a decreased TOPO-II expression, while Kim et al.⁷ showed that TOPO-II remained unchanged and

demonstrated that only the mean expression level of MRP was higher than in normal kidneys. Dekel et al. claimed that higher TOPO-II expression was found in the more aggressive renal cell tumors, characterized by higher tumor grade and higher postoperative recurrence rates¹.

Correlation between resistance to chemotherapy and higher expression of these proteins was demonstrated in-vitro using RCC cell lines. Higher LRP expression was found to be strongly correlated with intrinsic resistance to chemotherapeutic drugs¹⁰. Moreover, adriamycin-resistant RCC cell lines which were established by long-term exposure to increasing concentrations of this drug, demonstrated higher contents of mRNA coding for MRP and lower content of TOPO-II mRNA in comparison to native cell lines²⁰. In contrast to the numerous extensive studies performed on RCC's, there is a paucity of data concerning multidrug resistance in urothelial carcinomas of the renal pelvis and ureter. Kong et al.⁸ reported that LRP was up-regulated in urothelial carcinomas, while MRP1 showed no similar extent of overexpression in these tumors. Koren et al.⁹ reported that Topo-II nuclear staining was positive in almost all transitional cell carcinoma samples studied and suggested that its expression could be thus of prognostic value in bladder carcinoma.

The modern multi-modality therapeutic approach to WT, combining surgery with radiotherapy and chemotherapy, results in high cure rates even in advanced stage disease, with a pivotal role attributed to chemotherapy. However, there are still cases which exhibit either primary or secondary drug resistance with dismal outcomes. The aim of our study was hence to measure the expression of representative MDR proteins (MRP-1, LRP and TOPO-IIa) in WT, using the tissue microarray technique. This technique not only has economical advantages compared to the standard histological methods, but it is also highly efficient, accurate and provides remarkable standardization of staining, as well as preservation of the original tissue¹³.

Review of the literature reveals that conflicting results have been published for TOPO-II and very few have studied the expression of the other drug resistance markers in WT's. Granzen et al.⁶ found that TOPO-II is predominantly expressed in the epithelial components of WT specimens, with no relation to chemotherapy treatment. On the other hand, in two microarray expression studies, TOPO-II was found to exhibit high expression in WT and was even suggested to be related to the chemosensitivity of the tumor^{15, 16}. In a recent publication, TOPO-II was likewise found to be overexpressed (but in anaplastic WT samples) and its increased expression correlated with WT tumor aggressiveness¹⁷. TOPO-II has been proposed as a diagnostic marker distinguishing mesoblastic nephromas from Wilms' tumors¹⁵. Blastemal and epithelial LRP expression in WT was reported by Efferth et al.² to correlate with tumor stage and previous exposure to chemotherapy. In a study which analyzed the expression level of MRP-1 in nephroblastomas, the latter was found to exhibit heterogenous expression, with significant relationship to patients' survival³.

Our study has further investigated the expression of these three different proteins associated with multidrug resistance in WT. All the tested markers exhibited some changes in their expression pattern in WT compared to normal kidney. The most prominent reduction in expression was observed for MRP-1 which is strongly expressed in normal kidneys and was very weakly expressed or unexpressed in more than 50 % of the WT samples studied. Similarly, LRP expression which was lost in the WT samples is weakly expressed in more than 50 % of normal kidneys. The reduction in expression of both these proteins associated with multi drug resistance in WT, suggests that these tumors are expected to be responsive to chemotherapy, and therefore these 2 markers could be positive indicators.

TOPO-IIa is the only gene found by us to be overexpressed in WT (in 21 % of samples) compared to normal kidney.

The differences between the expressions of the studied proteins in the authentic tumors and in their related xenografts suggest that changes in expression may occur during the establishment of the xenografts in nude mice. Indeed, comparison of expressions of MRP-1 as well as MDR-1 in paired cell lines derived from primary and

metastatic RCC also revealed that the drug resistance phenotype of the primary tumors may not necessarily reflect that of their metastases, implying possible changes during the formation of the metastases⁴. Taken together, interpretation of studies looking at the response to chemotherapy in animal models may be difficult and misleading.

All the proteins studied here were exclusively expressed in the tubular component in some of the tumors and were either not or very weakly expressed in the blastemal component of the tumor. This unexplained observation may have prognostic significance in accord with the International Society of Paediatric Oncology (SIOP) classification which refers to blastemal nephroblastoma as a high risk tumor¹⁹. Understandably, in order to better evaluate the prognostic relevance of these markers we would have had to compare their expressions in WT tissues obtained pre and post-chemotherapy, but as we routinely follow the recommendations of the National Wilms' Tumor Study Group (NWTSG) advocating early surgery, such evaluation was not feasible. Another limitation of the study which should be acknowledged is the lack of data on the clinical outcome of the patients and therefore the clinical implications of our findings are still vague and warrant further research.

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