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Gene expression analysis of blastemal component reveals genes associated with relapse mechanism in Wilms tumour

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ABSTRACT

Wilms tumour (WT) is a paediatric kidney tumour, composed of blastemal, epithelial and stromal cells, with a relapse rate of approximately 15%. Long-term survival for patients with relapse remains approximately 50%. Current clinical and molecular research is directed towards identifying prognostic factors to define the minimal and intensive therapy for successful treatment of children with low and high risk of relapse, respectively. Blastemal component presents a high level of aggressiveness and responsiveness to chemotherapy. To identify molecular prognostic markers that are predictive of chemotherapy sensitivity in tumour relapse, blastemal-enriched samples from stage III and IV WT, from patients with relapse or without relapse, were analysed for 4608 human genes immobilised on a customised cDNA platform. These analyses revealed 69 differentially expressed genes, and the top nine genes were further evaluated by qRT-PCR in the initial WT samples. *TSPAN3*, *NCOA6*, *CDO1*, *MPP2* and *MCM2* were confirmed to be down-regulated in relapse WT, and *TSPAN3* and *NCOA6* were also validated in an independent sample group. Protein expression of MCM2 and NCOA6 were observed in 38% (13 out of 34) and 28% (9 out of 32), respectively, of independent stage III and IV WT blastema samples, without association with relapse. However, a significant association between MCM2 positive staining and chemotherapy as first treatment suggests the involvement of MCM2 with drug metabolism in WT blastemal cells.

1. Introduction

Wilms tumour (WT), or nephroblastoma, is the most common type of malignant renal tumour in childhood, affecting one in 10,000 children. Approximately 75% of the cases occur in children younger than five years old, with a peak incidence in two to three year olds.[1]

WT has a classical triphasic histology that recapitulates foetal kidney, with varying proportions of blastemal, epithelial and stromal cells.[2,3] Tumour stage and histologic subtype

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have long been recognised as important prognostic factors, and they are currently used for therapeutic approaches.[4] The most important adverse prognostic indicator with respect to histology in untreated WT is the presence of anaplasia that accounts for only 5% to 10% of all cases. In chemotherapy pretreated tumours, the persistence of the blastemal component has also been implicated as a marker of poor outcome.[5]

Overall relapse rate for children with WT is around 10% to 15%[6] and their prognosis depends on several factors, such as initial stage, site of relapse, time from initial diagnosis to relapse and previous therapy.[7,8] Approximately 50% of the patients with relapse are going to be cured with modern therapy combinations.[9,10] Clinical and molecular researches are focusing on defining the minimal therapy for successful treatment of WT, limiting the late effects of treatment while maintaining an excellent survival rate.[11–13] Loss of heterozygosity (LOH) of both 1p and 16q in the same sample predicts an increased risk of relapse and death and has been contributing for clinical decisions by Children Oncology Group (COG). However, LOH is detected in a very small subset of WT patients, making this feature a less sensitive prognostic factor.[14,15]

Expression profiles have been used to assess the mechanisms involved in chemotherapy resistance and to identify useful biomarkers in assisting with patient stratification.[11–13,16] These analyses have been performed using all WT components, with different proportions of blastemal, epithelial and stromal cells. Recently, we demonstrated that the three histological components harbour distinct expression profiles with different levels of similarity to the earliest stages of kidney differentiation. Blastemal cells reported to have the most similar expression profile, suggesting that these cells contain the first molecular alterations that disrupt foetal kidney differentiation and provide permissive conditions for WT onset.[17] Therefore, we reasoned that the assessment of molecular divergence in blastemal components from WT with distinct responses to standard treatment might be a more sensitive approach to identify biomarkers useful for patient stratification regarding relapse.

2. Material and methods

2.1. Samples

For mRNA expression analysis, frozen samples from sporadic favourable histology WT were received from the Children Oncology Group (COG). Patients were enrolled in the National Wilms Tumour Study (NWTS)-5. Blastemal component was isolated from 57 blastemal predominant WT samples from primary nephrectomy at the time of initial diagnosis. Twenty-six advanced stage (III or IV) WT samples were used for the initial experiments, of which 13 samples were from patients without relapse after a follow up of at least 3 years. The remaining 31 WT samples were used as an independent group for biological validation, of which nine were from patients with relapse. All WT had their blastemal histological component isolated using a scalpel and only samples with >80% of blastemal cells were used for microarray and quantitative RT-PCR experiments.

For immunohistochemistry analysis, a tissue-microarray (TMA) containing 34 blastemal WT components from stages III and IV and 20 differentiated kidney samples was obtained from the archives of the Department of Anatomic Pathology, A.C. Camargo Hospital, São Paulo, Brazil. From 34 tumour samples, 11 were obtained from patients who received preoperative chemotherapy, 22 from patients with primary surgery and one from a patient for whom no information about treatment was available. For NCOA6 and MCM2 proteins, 32 and 34 samples presented confident immunohistochemistry staining and were analysed, respectively.

This study is in accordance with the principles of the Declaration of Helsinki and has been approved by the ethical committee from the A.C. Camargo Hospital under number 764/06.

2.2. RNA extraction and quality criteria

Total RNA was isolated using TRIzol (Sigma) according to the manufacturer's instructions. RNA quantity and integrity were assessed by spectrophotometry (Nanodrop) and microfluidics-based electrophoresis (Bioanalyzer, Agilent 2100), respectively. Only RNA samples with OD of approximately 2.0 and RIN >5.0 were used for quantitative RT-PCR and for microarray experiments.

2.3. Microarray experiments

A two-round RNA amplification procedure was carried out for all samples as described.[17] Labelled cDNA was generated in a reverse transcriptase reaction (IMPROM-PROMEGA) using 4 μg of amplified RNA (aRNA), random hexamer primer (Invitrogen Life Technologies, Carlsbad, CA), Alexa-3- or Alexa-5-labelled dCTP (Amersham, Biosciences). Five micrograms of test and reference cDNA reverse colour Alexa-labelled aRNA targets were competitively hybridised against the cDNA probes in a customised cDNA platform with 4608 ORESTES representing human genes.[18] Dye-swap was performed for each sample as control for dye bias and used as replicate. Pre-hybridisation and hybridisation were performed as described.[17]

A pool of RNA obtained from 15 different human cell[19] lines was used as reference. Signal intensities capture and analysis were performed as described.[17]

Experiments were submitted to GEO under number GSE22696 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22696).

2.4. Quantitative RT-PCR (qRT-PCR)

For qRT-PCR reactions, total RNA (RIN $\geqslant$ 5.0) was converted into cDNA in the presence of reverse transcriptase (IMPROM-PROMEGA) and oligo-dT[18] (PROMEGA – IDT). Reactions were performed on an ABI Prism 7900 sequence detection system (Applied Biosystems) using SYBR® Green PCR Master Mix (Applied Biosystems) in a total volume of 20 μl. Amplification efficiency of each primer pair was calculated using standard curve dilutions and specificity of fragment amplification was verified through dissociation curves (Table 1). No-template reactions were used as negative controls.[17] *ACTB* and *GAPDH* were identified by GeNorm[20] as the

Table 1 – Assayed genes with corresponding primer sequences and efficiencies.

Gene symbol	Annotation	Primers sequence	Amplified fragment (pb)	Efficiency (%)
MCM2	Minichromosome maintenance complex component 2	F CAGCAGGACACTATTGAGG R GCAGAGAGGTTGTGGATG	77	90
TSPAN3	Tetraspanin 3	F CATGTGATCTGGGCCG R CGCCAGTGATGAGGAG	118	89
NCOA6	Nuclear receptor coactivator 6	F CTGAAAGTGTGGAAAATGG R CCTGCTTGTTTACTTGG	122	94
ARPC5	Actin related protein 2/3 complex, subunit 5	F GGATTTGAGAGCCCGTC R GACCCTACTCCTCCAG	83	91
MAP1B	Microtubule-associated protein 1B	F GAAGGAAAGGCTCAGTGG R GCTGTTTCTCATGGGTCTC	103	88
KNTC1	Kinetochore associated 1	F GCTTTGGAATGGACTCTTGC R GAATGGATACTGGAGATGGC	90	89
MPP2	Membrane protein, palmitoylated 2 (MAGUK p55 subfamily member 2)	F CGCTGGAGAGTGGAATATC R GGTCAAAGTAGTGCCCG	105	97
RGS16	Regulator of G-protein signalling 16	F CAGGGCACACCAGATC R GGTTCATCCTCGTCAGC	95	95
CDO1	Cysteine dioxygenase, type I	F CAGTCCACCTTTTGATAC R CTTAGTTGTTCTCCAGC	134	105

most stable control genes from four endogenous candidate genes (*GAPDH*, *ACTB*, *HPRT1*, and *BCR*) and used for data normalisation. *HOXA11*, previously selected by microarray results, was excluded from qRT-PCR experiments due to the impossibility of obtaining specific fragment.

Reactions using cDNA converted from total RNA of WT samples and of HEK293 cell line were performed in duplicate and triplicate, respectively.

2.5. Methylation analysis

A human embryonic kidney cell line, HEK293, was maintained in DMEM supplemented with 10% of foetal bovine serum, 1% penicillin/streptomycin. HEK293 was cultured for 5 days in the presence (treatment) or absence (control) of 5 uM of the DNA demethylating agent 5-aza-2′-deoxycytidine (Sigma–Aldrich) dissolved in 50% of acetic acid. Control and treatment were performed in duplicate.

Selected genes were analysed for the presence of CpG islands using the UCSC Genome Browser (http://genome.ucsc.edu/) and their expression were evaluated by qRT-PCR in control and treated HEK293 cells.

2.6. Immunohistochemistry assays

Immunostaining was performed in the TMA using a mouse monoclonal anti-MCM2 antibody from Abcam® (clone CRCT1, 1:1000, #ab1653) and a rabbit polyclonal anti-NCOA6 from Atlas® (clone 14, 1:25, #HPA004198), with antigen retrieval by incubation on citrate buffer pH 6. Detection was performed using Advance (DAKO) and Novolink Polymer (Novocastra). Negative controls were established by (a) incubating slides with PBS alone and (b) omitting primary antibody. Immunohistochemical reactions were performed in duplicate. Cases were classified as negative (⩽10% of positive tumour cells), weak positive (11–50% of positive tumour cells)- and strong positive (>50% of positive tumour cells). For protein association with clinical information, two categories were used: negative (absence of weak staining) and positive (strong staining).

2.7. Mathematical and statistical analysis

In cDNA microarray data, student *t*-test was used to identify differentially expressed genes between relapse and no-relapse WT ($p < 0.01$). TMEV[21] was used to perform non-supervised hierarchical clustering using Pearson correlation and average linkage. Differentially expressed genes were annotated in biological process categories according to Gene Ontology Database and organised in a hierarchical manner through automated analysis of gene function of EASE.[22]

For qRT-PCR experiments, relative fold changes were calculated using Pfaffl model.[23] Genes were considered differentially expressed between the groups when the difference was $\geqslant|1.5|$ and p-value <0.05 (t student test).

To compare immunohistochemistry data and clinicopathological variables, Pearson's chi-square and Fisher's exact tests from SPSS 17.0.2 software were used.

3. Results

3.1. Identification of differentially expressed genes between relapse and no-relapse Wilms tumour samples

Gene expression profiles of 26 blastemal-enriched WT samples were compared (13 relapse and 13 no-relapse), resulting in 69 differentially expressed genes ($p < 0.01$). Of these, 29 were down-regulated, and 40 were up-regulated in relapse WT group (Supplemental Table 1), with fold change ranging from −2.02 for *HOXA11* to 1.74 for *RGS16*. Non-supervised hierarchical clustering of these 69 genes was not able to correctly discriminate between relapse and no-relapse WT samples, indicating a highly similar expression pattern of blastemal components between the two WT sample groups.

Forty-eight out of 69 genes were able to be annotated into Gene Ontology categories. Only up-regulated genes in WT re-

Table 2 – Classification of the differentially expressed genes between relapse and no-relapse WT samples within the biological process categories.

	Up-regulated genes in relapse WT	Down-regulated genes in no-relapse WT
Cell adhesion and migration	*CTNNA1, PRG2, ARPC5, TTN*	–
Apoptosis, cell proliferation and growth	*HDGFRP3, KNTC1, PPP1R9B, TCF8, RAB17, SORCS2, TRIM14, CASP4*	*ELN, EHHADH, MCM2, NCOA6, VPS28*
Signal transduction	*P2RY1, PPP1R9B, RGS16*	*DIRAS1, MPP2,*
Transcriptional regulation	*CARS, KIAA0368*	*ARNT2, HOXA11, PRKCBP1*
Metabolism	*KIAA0323, NOS3, PLA2G6, STK10, UGCGL1*	*ACP5, UBAP1, DCLRE1A, DDX24, HPGD, DUOX1*
Miscellaneous	*GOLPH3, PLA2G6, GPM6B, KIAA0913, SPRY4*	–
Unknown	*FGFBP1, TMEM63A, PPP1R15B, PARP12, UTP15, MIER1, KLHDC5, KIAA1602, HBEGF, CLPB, RAB3GAP2, TBRG1, CLINT1, ANGEL2, MAP1B*	*C7orf36, C20orf30, AK023371, ACAD9, ZSCAN21, KCTD21, ANKMY2, RNF11, MOBKL2B, UBLCP1, PRKD3*

lapse group were classified in cell adhesion and migration category. The other categories contained both up- and down-regulated genes, without any detectable preferential distribution (Table 2).

Subsequently, the top nine differentially expressed genes (*ARPC5*, *MAP1B*, *KNTC1*, and *RGS16* are up-regulated in relapse WT; *TSPAN3*, *CDO1*, *NCOA6, MPP2*, and *MCM2* are down-regulated in relapse WT) were selected and used for technical validation by qRT-PCR. *TSPAN3*, *CDO1*, *NCOA6*, *MPP2*, and *MCM2*, all down-regulated in relapse WT, were in agreement between both microarray and qRT-PCR methods.

3.2. *Expression control analysis of the technically validated genes by methylation*

To verify if the technically validated genes (*TSPAN3*, *MPP2*, *CDO1*, *MCM2* and *NCOA6*) were under methylation control, we first verified the presence of CpG islands in their 5′ end. All of them showed a potential region that fulfilled CpG island criteria. Then, expression level was assessed in HEK293 cells before and after treatment with a demethylating agent. No increase in expression level was detected suggesting no methylation control for these genes in this cell line.

3.3. *MCM2 and NCOA6 protein expression in Wilms tumour*

Protein expressions of MCM2 and NCOA6 were assessed by immunohistochemistry in an additional group of 34 stage III and IV blastemal-enriched WT samples from patients with different treatment protocols and 20 differentiated normal kidneys.

Experiments with MCM2 and NCOA6 showed nuclear staining. Among the 34 WT samples assessed for MCM2, 14 and 7 WT samples showed complete absence and weak staining, respectively; these were categorised as negative (61.8%). Strong positive staining was observed for 13 samples (38.2%). MCM2 in differentiated kidneys showed complete absence (17 out of 20) or very occasional weakly positive cells (3 out of 20) (Fig. 1A–C).

Among the 32 samples evaluated for NCOA6, 7 and 16 WT samples showed complete absence and weak staining, respectively; these were categorised as negative (71.9%). Strong positive immunostaining was observed in nine samples (28.1%). Differentiated kidneys were positive for NCOA6 in the majority of the cells (Fig. 1D–F).

Subsequently, association of MCM2 and NCOA6 protein expressions with clinicopathological variables was investigated (Table 3). Neither MCM2 nor NCOA6 reported association with relapse but MCM2 expression was associated with preoperative chemotherapy.

MCM2 protein expression was detected in 64% of tumours from patients who received preoperative chemotherapy against 27% of tumours from patients who underwent surgery as primary treatment, suggesting that MCM2 protein expression is modulated by chemotherapy exposure.

3.4. *Evaluation of differentially expressed genes in an independent set of WT samples*

To assess the five technically validated genes (*TSPAN3*, *CDO1*, *NCOA6*, *MPP2*, and *MCM2*) in a wide range of samples with different biological background, we tested them by qRT-PCR in an independent group of 31 blastemal-enriched WT samples, of which 9 and 22 were obtained from relapse and no-relapse patients, respectively.

NCOA6 and *TSPAN3* were down-regulated in relapse WT samples with statistical significance, in agreement with the initial sample group, showing fold changes of −6.4 and −2.6, respectively. The three remaining genes showed no difference in expression level in independent sample groups, probably due to the heterogeneous biological background of WT samples (Fig. 2).

Although NCOA6 and TSPAN3 showed to be differently expressed, these genes by themselves could not discriminate samples according to relapse status since there is a large overlap in the relative gene expression values of the samples in both groups. Therefore, in an attempt to use them as a molecular tool able to predict relapse, relative gene expression of both genes was combined with the expression value of the validated genes in the initial sample set (*MPP2*, *MCM2* and *CDO1*) to create trios that may be used to classify the WT samples. Next, specificity and sensitivity in discriminating relapse and no-relapse samples were determined (Table 4). The best

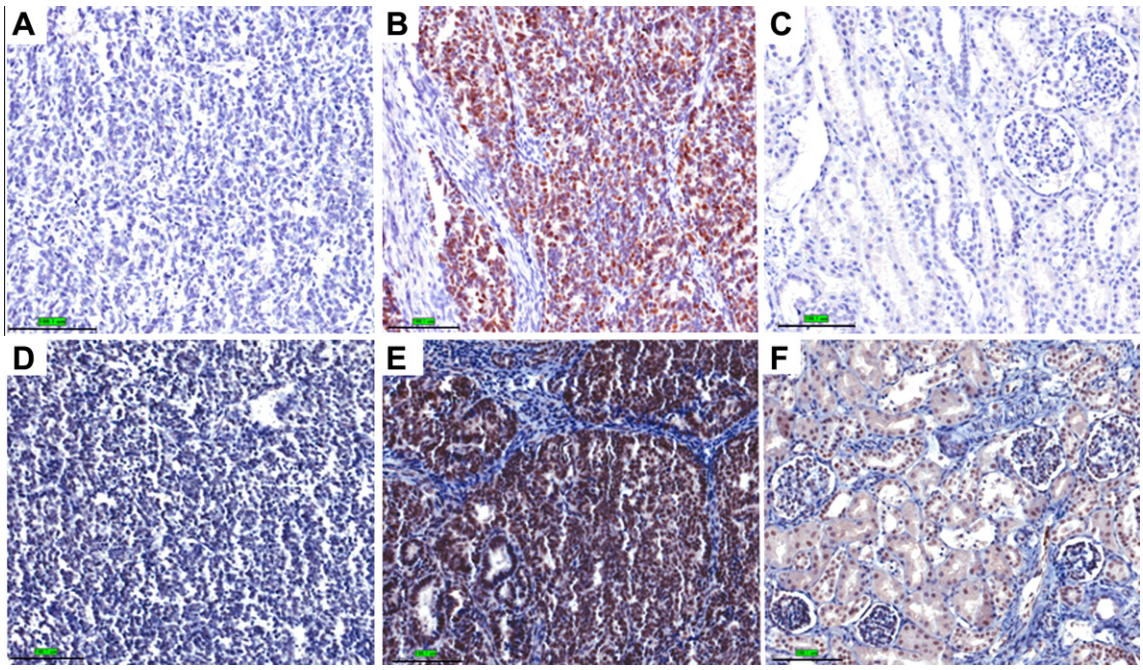

Fig. 1 – Representative sections with immunohistochemical staining of MCM2 and NCO6 in blastemal components of WT. (A–C) MCM2 staining. (A) negative WT blastemal cells. (B) positive WT blastemal cells. (C) negative differentiated kidney. (D–F) NCOA6 nuclear staining. (D) negative WT blastemal cells. (E) positive WT blastemal cells. (F) differentiated kidneys positive for NCOA6. Black bars represent 100.1 μm as indicated in the green box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3 – Association of MCM2 and NCOA6 protein expression in WT samples (blastemal component) and clinicopathological variables.

	Category	MCM2			NCOA6		
		Negative (%)	Positive (%)	*P*	Negative (%)	Positive (%)	*p*
Clinical stage	III	16 (61.5)	10 (38.5)	0.65	17 (70.8)	7 (29.2)	0.60
	IV	5 (62.5)	3 (37.5)		6 (75.0)	2 (25.0)	
Gender	Male	7 (58.3)	5 (41.7)	0.52	10 (83.3)	2 (16.7)	0.24
	Female	14 (63.6)	8 (36.4)		13 (65.0)	7 (35.0)	
Average age	<3 years	2 (40.0)	3 (60.0)	0.27	3 (75.0)	1 (25.0)	0.69
	>3 years	19 (65.5)	10 (34.5)		20 (71.4)	8 (28.6)	
Pre-operative chemotherapy	No	16 (72.7)	6 (27.3)	0.05*	14 (73.7)	5 (26.3)	0.49
	Yes	4 (36.4)	7 (63.6)		8 (66.7)	4 (33.3)	
Relapse	No	14 (63.6)	8 (36.4)	0.65	16 (76.2)	5 (23.8)	0.52
	Yes	7 (63.4)	4 (36.4)		7 (70.0)	3 (30.0)	
Death	No	13 (59.1)	9 (40.9)	0.35	15 (75.0)	5 (25.0)	0.61
	Yes	8 (72.7)	3 (27.3)		8 (72.7)	3 (27.3)	

trio comprised the *TSPAN3*, *NCOA6* and *CDO1* that could correctly discriminate 81.8% of no-relapse and 100% of relapse samples with a sensitivity of 77.8% and a specificity of 100%.

4. Discussion

Despite the efforts to identify a more sensitive molecular marker useful for favourable histology WT prognosis, tumour stage remains the most significant prognostic variable widely used in treatment regimens, separating WT in two distinct subgroups: patients who require minimal therapy and patients who require modified therapy regimen. Currently, the only molecular analysis, used by COG, for this stratification is based on genomic observation. Genomic losses of both 1p and 16q are considered adverse risk factors.[14,15,24] The current goal is to find additional molecular markers to offer for patients with low risk of relapse a therapy that minimises the potential late toxicities, without decreasing the current survival rates.[25]

To identify molecular markers, some researchers have performed studies to establish an association between gene expression and WT prognosis by using all components of the tumour.[12,13,16,26,27] However, because the blastemal component is more responsive to chemotherapy, and because this

Gene	Fold microarray	Fold qRT-PCR Initial set	Fold qRT-PCR Independent set
RGS16	1.7*	-1.1	-
MAP1B	1.5*	1.5	-
KNTC1	1.5*	-1.4	-
ARPC5	1.5*	-1.1	-
TSPAN3	-1.5*	-1.8*	-2.6*
MPP2	-1.5*	-2.1*	-2.0
MCM2	-1.5*	-1.6*	-1.6
NCOA6	-1.6*	-1.7*	-6.4*
CDO1	-1.6*	-1.2*	1.5

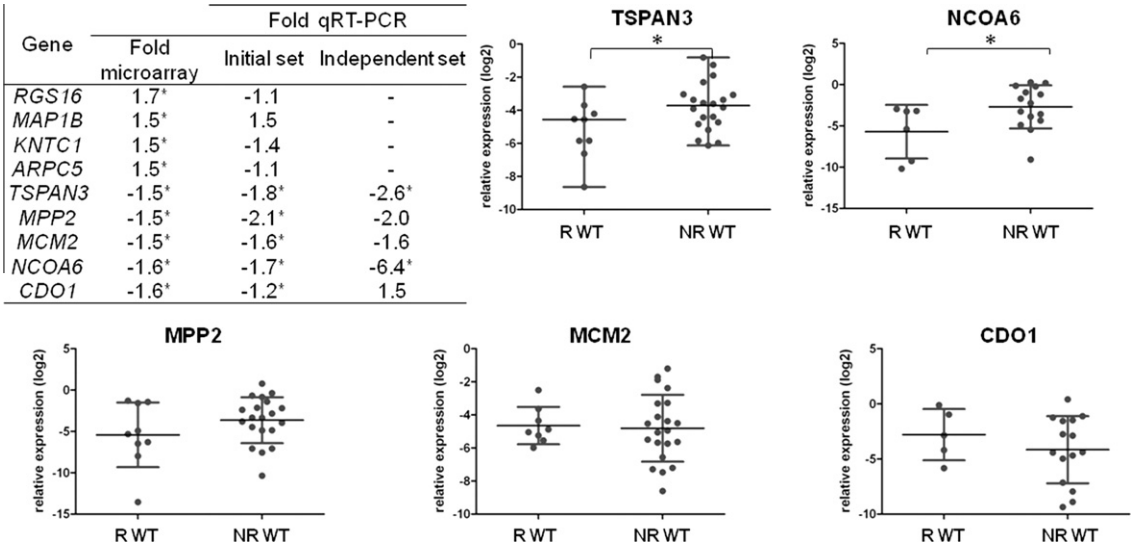

Fig. 2 – Comparison of gene expression between relapse and no-relapse WT samples. Fold-change from microarray and qRT-PCR experiments in initial and independent sample sets is presented in the table. Graphics illustrate relative expression of *TSPAN3*, *NCOA6*, *MPP2*, *MCM2*, and *CDO1* in the independent sample set. (*) Genes considered differentially expressed with statistical significance ($p \leqslant 0.05$). Negative and positive values correspond to down- and up-regulation in relapse WT, respectively. R WT – relapse WT samples. NR WT – no-relapse WT samples.

Table 4 – Prediction of no-relapse and relapse WT samples through expression of trios of genes.

	NR (%)	R (%)	Sensitivity (%)	Specificity (%)
NCOA6, TSPAN3, MPP2	10/12 (83.3)	6/7 (85.7)	6/8 (75)	10/11 (90.9)
NCOA6, TSPAN3, MCM2	10/13 (76.9)	4/7 (57.1)	4/7 (57.1)	10/13 (76.9)
NCOA6, TSPAN3, CDO1	9/11 (81.8)	7/7 (100)	7/9 (77.8)	9/9 (100)

component retains the initial molecular alteration critical for WT onset,[17] it seems reasonable that the comparison between gene expression patterns of blastemal component samples from relapse and no-relapse patients can reveal more promising molecular factors involved in the relapse mechanism in WT. In this study, we used relapse and no-relapse blastemal-enriched WT samples to assess their gene expression profiles. As a direct consequence of the proposed experimental design, the gene expression experiments are less influenced by the typical wide spectrum of different cell types present in the tumour. This may lead to a higher sensitivity to detect small differences in gene modulation and a higher chance to be significantly associated with relapse. This concept was partially confirmed in this study by the low level of difference in fold-change that was reported by the microarray experiment with a reasonable validation rate of 56% in qRT-PCR methodology.

TSPAN3 (15q24.3), *NCOA6* (20q11), *MPP2* (17q12–q21), *CDO1* (5q22–q23) and *MCM2* (3q21), the five genes with expression differences confirmed in the initial sample set, were down-regulated in the relapse sample group. Moreover, results of *TSPAN3* and *NCOA6* were validated in an independent group of samples, making these genes the most important candidates to be investigated in the mechanism of relapse in WT.

These two genes by themselves could not discriminate the distinct groups in the samples, probably due to the dependency of the relapse mechanism on many others molecular factors.

TSPAN3 was not previously associated with tumours and little is known about its function. *NCOA6* has the potential to enhance the activity of a wide variety of other transcription factors, including *cFOS*, *cJUN*, *CREB*, *NFKB*, *ATF2*, heat shock factors, *E2F1*, *SRF*, *RB*, *TP53* and *SOX9*. Thus, *NCOA6* may function as a transcriptional co-integrator of important transcription factors involved in growth, proliferation, cytokine signalling, metabolism and apoptosis.[28] *CDO1* promoter methylation was shown to be a predictor for distant metastasis in a specific group of breast cancer.[29] The role of *MPP2* in cancer still need to be investigated. *MCM2* was previously showed by us to be up-regulated at the transcription level in WT samples when compared to a pool of foetal kidneys.[17] Over-expression was also shown by others at the protein level in renal cancer.[30,31]

The present work showed that MCM2 transcription level is also involved in the relapse mechanism in WT. Evidences showed that MCM2 proteins are present only during the cell cycle and, thus, are specific markers of proliferating cells.[32] In addition, the presence of MCM2 protein was associated with WT blastemal component from patients who received chemotherapy before surgery. Whether this association reflects the rate of proliferation in blastemal cells, and/or their reaction over the chemotherapy treatment remains to be ad-

dressed. In any case, modulation of MCM2 at the mRNA and protein levels suggests an association between MCM2 expression and relapse mechanism in WT, which is probably mediated by chemotherapy treatment.

The advantage of DNA over RNA molecules for clinical proposals is evident. In this regard, we evaluated whether the expression regulation of the candidate genes were under methylation control to verify the possibility of transferring the information acquired from mRNA expression to analysis of DNA CpG methylation. Unfortunately we did not observe expression reactivations of *TSPAN3*, *CDO1*, *NCOA6*, *MPP2* and *MCM2* in HEK293, an embryonic kidney cell, after treatment with demethylating agent, even for *CDO1* previously found to be differentially methylated in breast tumour samples.[29] If genes are not methylated in other cell type or if expression regulation is not under methylation control at all, remains to be addressed.

Altogether, in spite of the relatively small number of samples evaluated in this study, the evidence shown here surely pointed to novel genes as being involved in relapse mechanism in WT. However, it is absolutely necessary to validate these candidates in a broader cohort with proper experimental delineation to use as molecular markers for stratifying patients for more individualised therapy regimen.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.05.024.

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