Regulation of p53 isoform expression in renal cell carcinoma

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1. ABSTRACT

Differential expression of p53 isoforms might participate in the marked resistance towards conventional chemotherapy of renal cell carcinomas (RCCs). Therefore, we analysed their differential expression and regulation in RCCs. RCCs expressed a more p53 activating isoform pattern during tumor initiation and progression, *in vivo*. *In vitro,* two cell lines exhibiting a similar sensitivity towards Topotecan-induced cell death revealed a similar induction of p53 target genes but strongly differed in their extent of apoptosis. Furthermore, they strongly differed in their basal expression patterns and differential regulation of the isoforms. In conclusion, our study examined for the first time the differential expression and regulation of all p53 isoforms in a tumor *in vivo*. Furthermore, novel results in our *in vitro* studies show that p53 isoforms are strongly differentially regulated by chemotherapy in RCCs and that expression and regulation of so-called "p53-target genes" are obviously at least in part regulated by other transcription factors. In addition, our original findings show that p53 isoform expression in RCC cell lines is of minor importance for sensitivity towards chemotherapy.

2. INTRODUCTION

Renal cell carcinomas (RCCs) exhibit a marked resistance towards conventional chemo- and radiotherapy, which makes treatment in metastatic disease difficult (1, 2). This therapeutic resistance of RCCs is at least in part due to inactivation of p53, which - as "a guardian of the genome" - normally regulates gene transcription of multiple genes involved in cell cycle control, DNA-repair, replicative senescence as well as apoptosis. In contrast to other tumors, however, in which p53 function is suppressed by inactivating mutations in approximately 50 % of cases (3), p53 is mutated in only 10 % of RCCs (4, 5). Despite the expression of wildtype p53 in most RCCs, the p53 DNA-damage response in RCCs is disrupted at a certain point. Upon DNA-damage, p53 exhibits a normal stabilization, translocation to the nucleus and DNA-binding; however, the function of p53 as an activator of transcription is suppressed, and this is independent of Mdm2, MdmX and Arf. A tissue specific mechanism seems to be responsible for the suppression of p53 function in RCCs, which is due to the loss of an essential component of the p53-signalling pathway or overexpression of a p53 inhibitor (6).

Table 1. Tumor grade and gender distribution referred to the tumor stage of the RCC samples used for RT-PCR

	G1[2]	G2[2]	G3[2]	male[3]	female[3]
pT1[1]	3	8	4	10	5
pT2[1]	0	12	3	8	7
pT3[1]	0	9	6	9	6

[1] Tumor stage of the RCCs investigated [2] Differentiation grade of the RCCs investigated [3] Gender of the patients with the RCCs

Recently, several isoforms of p53 with either inhibitory or activating functions with respect to p53 dependent gene induction were identified. They comprise full length p53 (hereafter termed p53), p53beta, p53gamma, del40p53(alpha) (hereafter termed del40p53), del40p53beta, del40p53gamma as well as del133p53(alpha) (hereafter termed del133p53), del133p53beta and del133p53gamma (7, 8). The p53, del40p53 and del133p53 isoforms result from different transcriptional start sites in the N-terminal region of the p53 gene, whereas the alpha, beta and gamma variants are generated by alternative splicing of intron 9. Besides structural differences between the isoforms (for details see (7, 9)) they differ in their subcellular localization and function. p53, del40p53, del133p53, p53beta and p53gamma are mainly located in the nucleus. In contrast, del133p53beta is localized in the nucleus and cytoplasm whereas del133p53gamma is localized exclusively in the cytoplasm (7). Besides their different subcellular localization, the isoforms exert different effects on p53 mediated gene expression. Some p53 isoforms have been shown to influence the transactivation activity of p53, whereas other isoforms exhibit a function that is independent on p53. Thus, the transactivation-deficient del133p53 isoform seems to have a dominant inhibitory effect on the transcriptional activity of p53, whereas in the C-terminal truncated variant del133p53beta the dominant-negative function is lost (10). In contrast, p53beta can bind to the endogenous Bax and p21 promotors by itself and furthermore bind to p53 directly. Thereby, the transcriptional activity of p53 is enhanced on the Bax, but not on the p21 promotor (11). The function of p53gamma is not fully understood. However, p53gamma seems to be defective in apoptosis-induction (10). Like the del133 isoforms, the del40 isoforms are unable to activate transcription by themselves, since they do not harbour the first transactivation domain. However, they can form complexes with p53 and modulate p53-dependent gene expression. Thus, they act either as activators or inhibitors of p53 regulated gene induction depending on the ratio of del40p53 to p53, on the target gene and possibly also on the tissue examined (12). Furthermore, del40p53 can prevent p53 from degradation by Mdm2 (13).

Taken together, it is reasonable to assume that a deregulation of p53 isoform expression might participate in tumor initiation and progression (14) and this has been analyzed in melanomas, oral lichen planus (OLP), ovarian cancer, acute myeloid leukemia (AML) and squamous cell carcinomas of the head and neck (SCCHN) (14-18). In SCCHN and OLP p53 was expressed in all samples whereas p53beta was detected in the majority of samples. p53gamma, del133p53, del133p53beta as well as del133p53gamma were each expressed in a smaller fraction of cases (15, 18). In AML a shift from shorter isoforms towards full-length p53 expression could be observed after chemotherapy. However, these studies focussed on the expression of p53 and del133p53 isoforms, whereas del40p53 isoform expression was not analyzed. High expression of del40p53 and p53beta was observed in melanomas although there was an abnormal subcellular distribution of these p53 isoforms (14). In ovarian cancer del40p53 and del133p53 expression levels did not differ between stage 1 and stage 3 tumors (16).

In view of the potential role of deregulated expression of the p53 isoforms in p53 inactivation, in tumor initiation and progression as well as in therapy resistance in RCCs, we investigated the expression of p53 isoforms in RCCs *in vivo* and *in vitro*. Although a study on the expression of the p53 and del133p53 isoforms in RCCs has been published recently (19), that analysis determined neither del40p53 isoform expression nor p53 and del133p53 isoform quantification. Furthermore, the relevance of p53 isoform expression for therapy resistance still remains to be elucidated.

3. MATERIAL AND METHODS

3.1. Patients and specimens

Tissue samples of 45 RCCs of the clear cell type of different tumor stages and grades as well as 25 non-neoplastic renal tissues were obtained from patients who had undergone nephrectomy at the University Hospital of Duesseldorf (Table 1). No preoperative chemotherapy was performed. Histological examination of the respective tumor tissues revealed a tumor cell content of more than 80 % for each sample.

3.2. Cells and culture

The permanent RCC cell lines clearCa-6 and clearCa-3 (20) were maintained with Dulbecco´s modified Eagle´s medium (DMEM, Gibco), supplemented with 10 % fetal calf serum (FCS), penicillin and streptomycin, asparate/asparagine and HEPES buffer and cultivated at 37°C in an atmosphere with 5 % CO_2. To analyze the mRNA expression of the p53 isoforms and selected target genes, RCC cell lines were seeded at 5×10^5 cells/cm^2 on a culture dish in standard growth medium. After 24 h tumor cells were exposed to 1 microgram/ml Topotecan (Hycamtin, Glaxo Smith Kline) for 4 h or 12 h and compared to untreated controls. We chose Topotecan as an example of classical DNA-damaging drugs, since induction of cell death by this Topoisomerase I inhibitor has been extensively examined in multiple RCC cell lines (2).

3.3. RNA extraction

Total RNA was isolated by trizol-chloroform extraction. The integrity of all tested total RNA samples was verified by agarose gel electrophoresis and detection of the 18S/28S rRNA bands.

3.4. Reverse transcription of the p53 isoforms

cDNA synthesis was performed with "SuperScript™ II Reverse Transcriptase" (Invitrogen)

Table 2. Sequences of the gene-specific primers used for reverse transcription

Gene	Primer-sequence (5´- 3´)
p53alpha	GAG TCA GGC CCT TCT
p53beta	AAA GCT GGT CTG GTC
p53gamma	TCG TAA GTC AAG TAG
SDHA	CAT GTT CCC CAG AGC

Table 3. Sequences of the primers used for RT-PCR

Primer	Sequence (5´-3´)		T
	Forward	Reverse	53 °C
p53 up	GCC GCA GTC AGA TCC TA	CTG GGA GCT TCA TCT GGA	53 °C
del133 up	CAG GAG GTG CTT ACA CA	AGG GGA GTA CTG TAG GAA	53 °C
del40 up	CGA AAA TTC CAT GGG ACT GA	GGG GAC AGC ATC AAA TCA TC	
SDHA up	TGG GAA CAA GAG GGC ATC TG	CCA CCA CTG CAT CAA ATT CAT G	59 °C
PIDD up	CGC TCT TGC TGT CTC ACA AC	AGG TAG CGT GTC CAG CAG AT	
p21 up	GAG GCC CGT GAG CGA TGG A	CAG CGG CGC GTT TGG GTG G	60 °C
Bax up	GCT GTT GGG CTG GAT CCA AG	TCA GCC CAT CTT CTT CCA GA	
NOXA up	CCG GCA GAA ACT CTG AAT C	CGT GCA CCT CCT GAG AAA AC	58 °C

according to the manufacturer´s protocol. In brief, for gene-specific cDNA synthesis 5 microgram of total RNA were reversely transcribed in a final volume of 20 microliter containing 10 pmol/microliter sequence-specific primer (primer-sequences: see Table 2) and 1 mM dNTP mix. After incubation at 65°C for 5 min, 4 microliter 5 x First Strand Buffer, 2 microliter DTT and 40 U RNase OUT were added, followed by incubation at 42°C for 2 min. Afterwards 1 microliter Superscript II Reverse Transcriptase was added and incubation at 42°C for 50 min and at 70°C for 15 min followed. Synthesized cDNA was diluted 1:5 for real-time-PCR.

3.5. Reverse transcription of the p53 inducible genes

cDNA synthesis was performed with the "First Strand cDNA Synthesis Kit" (Fermentas) according to the manufacturer´s protocol. In brief, for cDNA synthesis 1 microgram of total RNA was mixed with 1 microliter random hexamer primers in a volume of 11 microliter. After denaturation at 65°C for 5 min 4 microliter 5 x reaction buffer, 1 microliter RiboLock RNase Inhibitor (20 u/microliter), 2 microliter 10 mM dNTP mix, and 2 microliter M-MuLV Reverse Transcriptase (20 u/microliter) were added, followed by incubation at 25°C for 5 min and at 37°C for 60 min. The reaction was terminated by incubation at 70°C for 5 min. Synthesized cDNA was diluted 1:5 for real-time-PCR.

3.6. Quantitative PCR

Amplification and quantification of the p53 isoforms p53, p53beta, p53gamma, del133p53, del133p53beta, del133p53gamma, del40p53, del40p53beta, del40p53gamma and the p53 target genes Bax, Noxa, PIDD and p21, as well as the housekeeping gene SDHA were carried out in a real-time PCR machine (Light Cycler, Roche Diagnostics) by using a total volume of 20 microliter, including 10 microliter SYBR Green qPCR SuperMix-UDG (Invitrogen), 4 microliter 5 x diluted cDNA, 1 microliter Bovine Serum Albumine (1 mg/ml stock solution), 1 micromolar each sense and antisense primer. The primer sequences used for amplification of the del40p53 isoforms were obtained from Marabese and coworkers (16). Real-time PCR was carried out with an initial incubation step at 50°C for 180 s, initial denaturation

at 95°C for 120 s and followed by 50 cycles for denaturation at 94°C for 5 s, annealing for 10 s at the primer-specific annealing temperature (see Table 3) followed by extension for 10 s at 72°C. A different qPCR protocol was used for amplification and quantification of PIDD: initial denaturation was performed at 95°C for 15 min followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 30 s and extension for 60 s at 72°C (21). Melting curves were directly drawn after amplification. All *in vitro* analyses were done in triplicates. PCR products were additionally checked by electrophoresis on 2 % agarose gels containing ethidium bromide and visualised under UV transillumination. PCR products were confirmed by DNA sequencing. To normalize the expression of the p53 isoforms and the p53-inducible genes, the housekeeping gene SDHA was used as an external standard. Statistical analysis of the mRNA expression in non-neoplastic renal tissue and RCCs *in vivo* was performed by the Mann-Whitney-Wilcoxon-test (SPSS 17.0) and in RCC cell lines *in vitro* by the Student´s T-test. P-values < 0.05 were regarded as statistically significant.

3.7. Western Blot analysis

Cell lines were lysed by exchanging cell culture medium with ice cold Lysis Buffer (150 mM NaCl, 30 mM Tris, pH: 7.5, 1 % Triton X-100, 10 % glycerine and protease inhibitors). Then, cells were scraped off the cell culture dish and the lysate was incubated for 10 min at 4°C. The lysates were centrifuged at 4° C at 13000 g for 10 min. Protein concentration of the supernatant was determined by the Bradford method (Biorad). Fifty microgram of protein lysate from cultured cells were separated under denaturing conditions in 7-10% polyacrylamide-gels. After blotting, the protein was transferred to a reinforced nitrocellulose membrane (Whatman GmbH) and equal loading of the proteins was checked by Ponceau Red staining. The membranes were then blocked with TBS containing 5% non-fat dry milk and 0.2 % Tween 20 for 24 h at 4°C. Afterwards the membranes were incubated for 2 h at room-temperature with human specific monoclonal or polyclonal primary antibodies (p53 from Calbiochem, mouse polyclonal: dilution 1:500, PARP from Cell Signaling, rabbit polyclonal, dilution 1:500 and beta-Actin from Sigma, mouse monoclonal, clone: AC-15, dilution 1:10000) and then washed 3 times for 5 min with TBS.
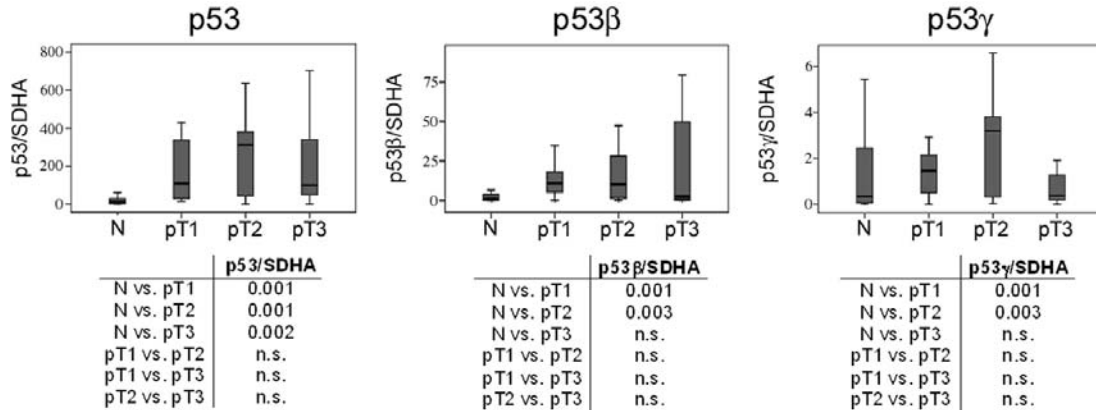
	p53/SDHA			p53β/SDHA			p53γ/SDHA
N vs. pT1	0.001		N vs. pT1	0.001		N vs. pT1	0.001
N vs. pT2	0.001		N vs. pT2	0.003		N vs. pT2	0.003
N vs. pT3	0.002		N vs. pT3	n.s.		N vs. pT3	n.s.
pT1 vs. pT2	n.s.		pT1 vs. pT2	n.s.		pT1 vs. pT2	n.s.
pT1 vs. pT3	n.s.		pT1 vs. pT3	n.s.		pT1 vs. pT3	n.s.
pT2 vs. pT3	n.s.		pT2 vs. pT3	n.s.		pT2 vs. pT3	n.s.

Figure 1. SDHA-normalized mRNA expression of the p53 isoforms in non-neoplastic renal tissue (N) and RCCs of different tumor stages. A significantly increased expression of p53, p53beta and p53gamma was detected in early stages and a significantly increased expression of p53 only in pT3-carcinomas.

The blots were incubated with a fluorescence-dye conjugated anti-mouse respectively anti-rabbit secondary antibody protected from light for 1 h at room-temperature and then washed 3 times for 5 min with TBS protected from light and scanned with the Odyssey Infrared Imaging System (LI-COR, Biosciences).

3.8. Caspase Assays

Detection of caspase-2, -3/-7, -8 and -9-like activities activation was performed with Colorimetric Assay kits according to the manufacturer's protocol (Biocat GmbH). In brief, untreated cells or cells treated with Topotecan (1 microgram/ml) for 24 h were harvested and lysed in 50 microliter chilled Cell Lysis Buffer for 10 min. Afterwards, the lysate was centrifuged for 3 min at 4000 g. The supernatant cytosolic extract was transferred to a fresh tube. Protein concentration of the supernatant was determined by the Bradford Method. Two-hundred and fifty microgram cytosolic extract were diluted to 50 microliter with cell lysis buffer. Fifty microliter of 2 x Reaction Buffer were added (containing 10 mM DTT). Caspase-specific substrate (caspase-2: VDVAD-pNA; caspase-3/7: DEVD-pNA; caspase-8: IETD-pNA; caspase-9: LEHD-pNA) was added to a final concentration of 200 micromolar and the mixture was incubated for 2 h at 37°C. Samples were read at 405 nm and extinction was compared to the untreated control. All analyses were done in triplicate.

3.9. Quantification of p53 isoform and p53 inducible gene expression

For relative quantification of the p53 isoforms and the housekeeping gene SDHA, serial cDNA dilution series were created for the p53, del133p53 and SDHA primers from clearCa-6 cDNA. The standard curve for the del40p53 primers was created from a cDNA pool of arbitrarily selected RCCs and non-neoplastic renal tissues. Serial cDNA dilutions for the SDHA standard curve ranged from 1/5 to 1/5000 ($r = 1.00$), for the p53 standard curve from 1/5 to 1/1000 ($r = 0.99$), for the del133p53 standard curve from 1/5 to 1/100 ($r = 0.97$) and for the del40p53 standard curve from 1/5 to 1/80 ($r = 0.98$).

For relative quantification of the p53 inducible genes serial dilution series were created from a clearCa-6 cDNA pool. Serial dilutions for Noxa and p21 ranged from 1/5 to 1/5000 ($r = 1.00$ for both), for Bax from 1/5 to 1/1000 ($r = 0.99$) and for PIDD from 1/5 to 1/500 ($r = 0.99$).

The specificity of the amplification reaction was confirmed by agarose gel electrophoresis, which revealed distinct bands for all PCR products, as well as by DNA sequencing.

4. RESULTS

4.1. p53, p53beta and p53gamma expression is upregulated in RCCs

Relative mRNA expression of the isoforms p53, p53beta and p53gamma (i.e. SHDA-normalized mRNA expression) was determined in all RCCs and non-neoplastic renal tissues. The relative mRNA expression of all three isoforms was significantly increased in RCCs confined to the kidney borders (pT1 and pT2) when compared to non-neoplastic renal tissue. Interestingly, expression of p53 was also upregulated in advanced RCCs infiltrating beyond the kidney borders (pT3) (Figure 1), whereas no significant alteration of the expression of p53beta and p53gamma in pT3 RCCs, as compared to non-neoplastic renal tissue, was detected. Relative mRNA expression of p53, p53beta and p53gamma revealed no significant differences between RCCs of the different tumor stages.

4.2. del133p53beta and del133p53gamma expression is downregulated in early tumor stages

Relative mRNA expression of the inhibitory del133p53 isoforms was determined in all RCCs and non-neoplastic renal tissues. No significant alteration of the expression of del133p53 could be detected in the different tumor stages, as compared to non-neoplastic renal tissue. However, a comparison of pT1 and pT3 RCCs revealed a significant upregulation of del133p53 expression in pT3 tumors. In contrast, the relative mRNA expression of del133p53beta and del133p53gamma was significantly decreased in pT1 and pT2 RCCs, as compared to non-neoplastic renal tissue, whereas no differences between pT3
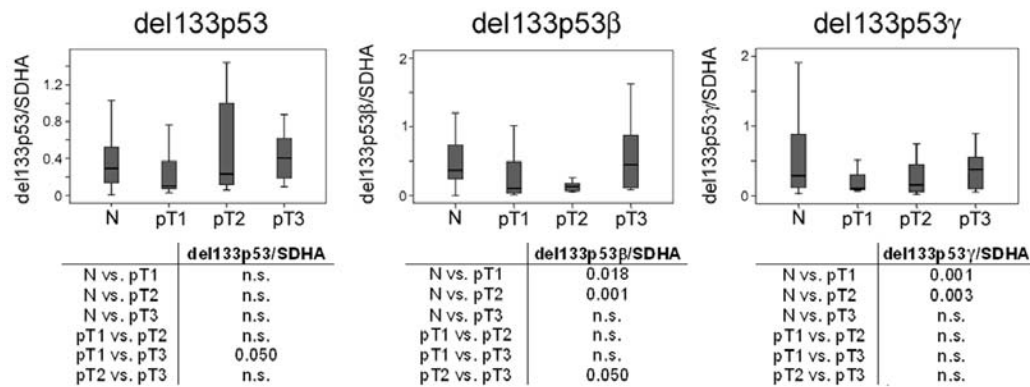
Figure 2. SDHA-normalized mRNA expression of the del133p53 isoforms in non-neoplastic renal tissue (N) and RCCs of different tumor stages. Significantly increased expression of del133p53 in pT3 RCCs compared to pT1 RCCs. Decreased expression of del133p53beta and del133p53gamma in pT1 and pT2 RCCs compared to non-neoplastic renal tissue.
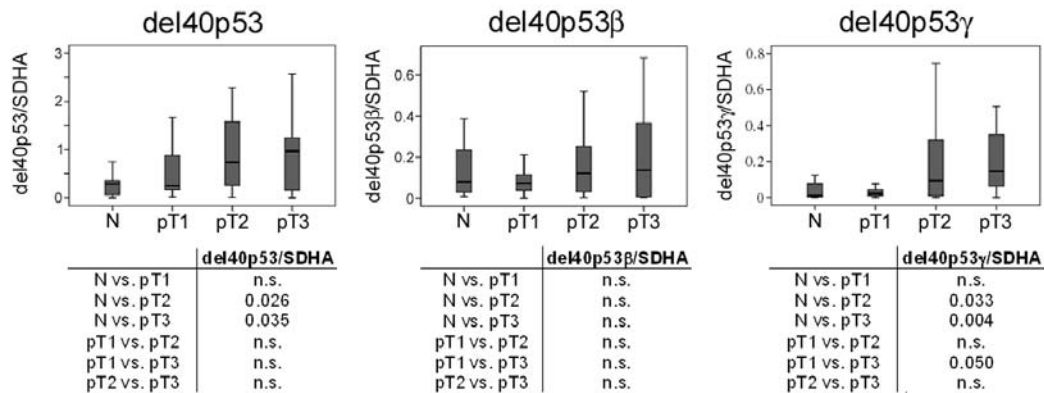

Figure 3. SDHA-normalized mRNA expression of the del40p53 isoforms in non-neoplastic renal tissue (N) and RCCs of different tumor stages. Significantly increased expression of del40p53 and del40p53gamma in pT2 and pT3 RCCs compared to non-neoplastic renal tissue. Furthermore, expression of del40p53gamma was significantly higher in advanced RCCs (pT3) compared to early tumor stages (pT1). No significant differences in del40p53beta expression throughout carcinogenesis and tumor progression.

RCCs and non-neoplastic renal tissue could be observed. Thus, expression of del133p53beta and del133p53gamma was significantly increased in pT3 tumors, as compared to pT2 tumors (Figure 2). Taken together, an overall decrease of del133p53 isoform expression could be observed in early tumor stages with no differences between advanced tumor stages and non-neoplastic renal tissue.

4.3. del40p53 and del40p53gamma expression is increased in late tumor stages

Expression of del40p53 and del40p53beta was detected in all RCCs and non-neoplastic renal tissues, whereas del40p53gamma could be detected in 19 out of 25 non-neoplastic renal tissues, 14 out of 15 pT1, 13 out of 15 pT2 and 13 out of 15 pT3 RCCs. The relative mRNA expression of del40p53 and del40p53gamma was significantly upregulated in pT2 and pT3 RCCs, as compared to non-neoplastic renal tissue. Furthermore, expression of del40p53gamma was significantly increased in pT3 RCCs when compared to pT1-carcinomas. In contrast, no

significant alteration of del40p53beta expression could be observed throughout carcinogenesis and tumor progression (Figure 3).

Taken together, our investigations surprisingly revealed an overall downregulation of the transcription inhibiting del133p53 isoforms in concert with an upregulation of mainly transcription activating p53 isoforms in early tumor stages. Furthermore, an overall increase of del40p53 isoform expression was observed during tumor progression.

4.4. Induction of p53 target genes in RCC cell lines exhibiting a similar sensitivity towards Topotecan-induced cell death

To further analyze and correlate p53 isoform expression to the transcriptional activity of p53 and chemotherapy sensitivity of RCCs we first examined the induction of typical p53 target genes in RCC cell lines exhibiting a similar sensitivity towards Topotecan-induced cell death (2) and then compared it to the expression of the
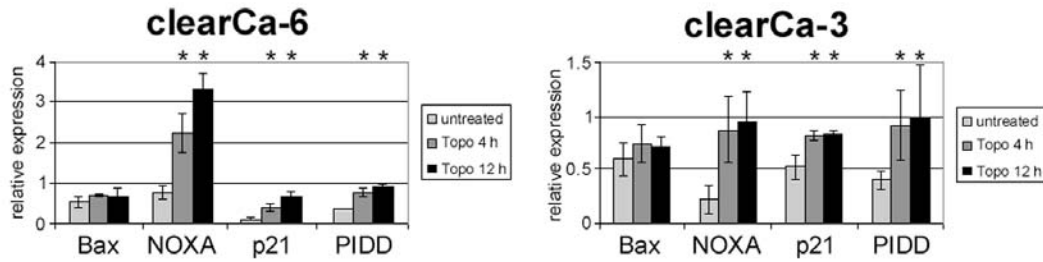
Figure 4. Transcriptional induction of p53 target genes in clearCa-6 and clearCa-3. The p53 inducible genes Noxa, p21 and PIDD were significantly induced 4 (Topo 4 h) and 12 h (Topo 12 h) after treatment with Topotecan (1 microgram/ml), whereas Bax was not induced in both cell lines (significant results are indicated *). The relative gene induction of Noxa and PIDD was similar in both cell lines (Noxa: 4.4 fold gene induction in both cell lines; PIDD: 2.7 fold gene induction in clearCa-6 and 2.5 fold gene induction in clearCa-3). p21 was induced stronger in clearCa-6 (6.9 fold gene induction) than in clearCa-3 (1.6 fold gene induction). (Shown are the mean and standard deviation of three independent experiments.)
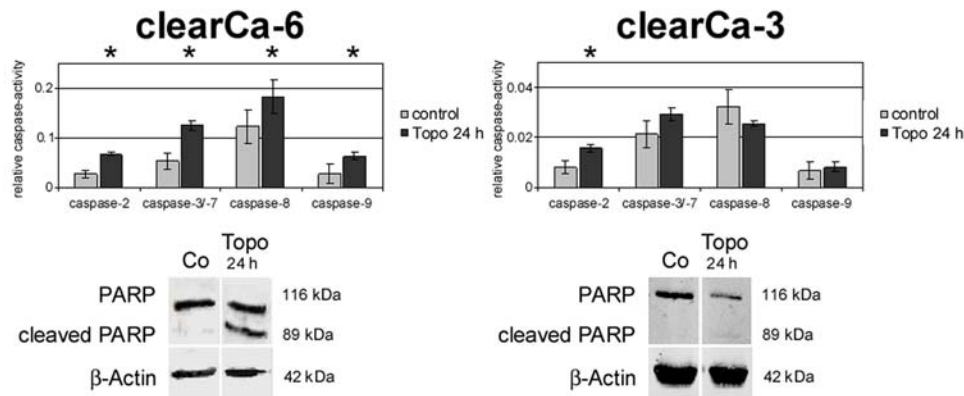

Figure 5. Induction of apoptosis in clearCa-6 and clearCa-3. Significant activation of caspases-2, -3/-7, -8 and -9 as well as strong PARP-cleavage after 24 h treatment with Topotecan (1 microgram/ml) (Topo 24 h) in clearCa-6. Significant activation only of caspase-2-like activities as well as barely detectable PARP-cleavage in clearCa-3 (* = significant caspase-activation). (Shown are the mean and standard deviation of three independent experiments.)

p53 isoforms. The RCC cell line clearCa-6, derived from a pT3 tumor, bears a mutation of the p53 gene at exon 8, codon 290, whereas clearCa-3, derived from a pT3 tumor as well, bears no p53 gene mutation (2). Interestingly, both cell lines exhibited a significant induction of the p53 inducible genes Noxa, p21 and PIDD after 4 and 12 h, whereas Bax was not induced in both cell lines (Figure 4). The extent of relative gene induction of Noxa and PIDD was similar in both cell lines (Noxa: 4.4 times gene induction in both cell lines; PIDD: 2.7 times gene induction in clearCa-6 and 2.5 times gene induction in clearCa-3). Only p21 was induced stronger in clearCa-6 (6.9 times gene induction) than in clearCa-3 (1.6 times gene induction).

4.5. Apoptosis induction by Topotecan in clearCa-6 and clearCa-3

Analysis of p53 target gene activation in the two cell lines revealed a very similar induction upon Topotecan treatment in clearCa-6 and clearCa-3. To examine if this correlated with similar apoptosis susceptibility, induction of apoptosis by Topotecan (1 microgram/ml) was compared

in clearCa-6 and clearCa-3 by colorimetric caspase assays as well as by analysis of PARP-cleavage. After 24 h incubation, clearCa-6 revealed a significant activation of caspase-2-, -8-, -9- and -3/7-like activities and a strong PARP-cleavage, whereas a significant activation only of caspase-2-like activities and a weak cleavage of PARP could be detected in clearCa-3 (Figure 5). Thus, induction of the p53 target genes investigated here could not predict sensitivity towards Topotecan-induced apoptosis.

4.6. Different basal expression levels of the p53 isoforms in clearCa-6 and clearCa-3

To test if the similar induction of p53 target genes in clearCa-6 and clearCa-3 correlated with a similar expression pattern of the p53 isoforms, we investigated mRNA expression in untreated cells. Here, clearCa-6 revealed a low basal expression level of all p53 isoforms. In clearCa-3 basal expression of p53, p53beta and p53gamma was approximately 400 times higher than in clearCa-6; moreover, clearCa-3 expressed the del40p53 isoforms approximately 1000 times higher than in clearCa-
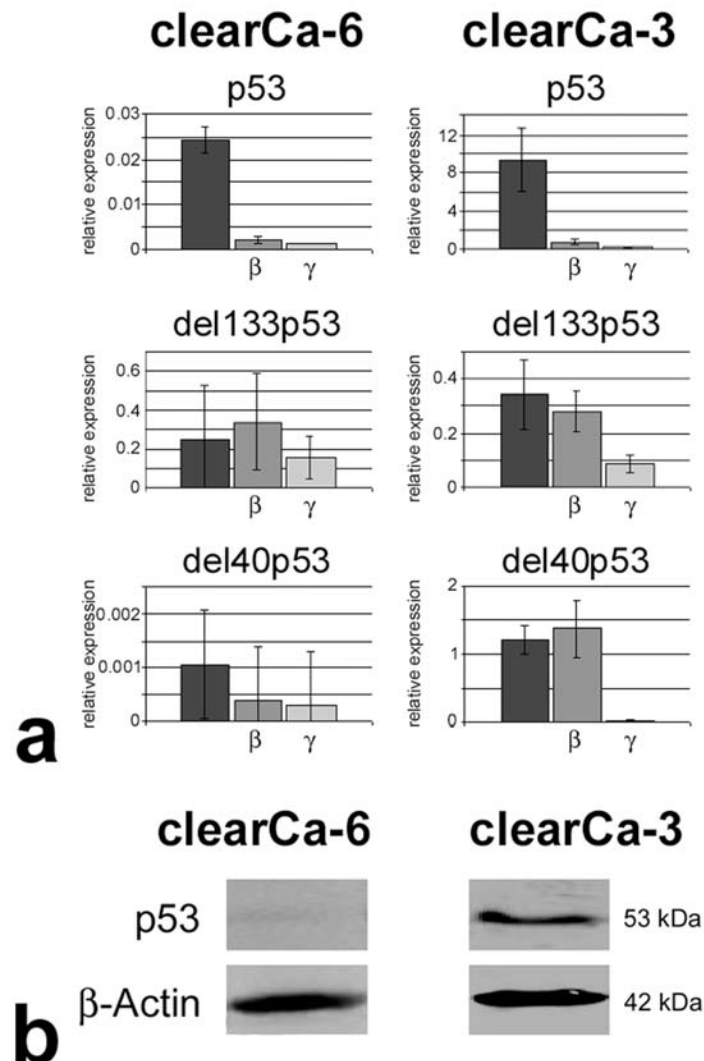
Figure 6. a) Relative expression of all p53 isoforms in untreated clearCa-6 and clearCa-3. All isoforms were expressed at a very low level in clearCa-6. In clearCa-3 basal p53 isoform expression was approximately 400 x higher and del40p53 isoform expression approximately 1000 x higher than in clearCa-6. del133p53 isoform expression did not differ. (Shown are the mean and standard deviation of three independent experiments.) b) The results made on the mRNA level by real-time PCR analysis and confirmed on the protein level by Western Blot for p53: In clearCa-6 p53 was barely detectable, whereas in clearCa-3 a strong p53 band became visible. (Shown are the mean and standard deviations of three independent experiments.)

6. The del133p53 isoforms were expressed to the same extent in clearCa-3 and clearCa-6 (Figure 6). In the absence of antibodies capable of recognizing each single isoform, we performed Western Blot experiments with a polyclonal antibody against p53 (7, 11). Figure 6 shows that just one band was detected for p53. The band was barely detectable in clearCa-6 but was strongly present in clearCa-3, thus confirming at the protein level the results made for p53 at the mRNA level.

4.7. Regulation of mRNA expression of the p53 isoforms in clearCa-6 and clearCa-3

Basal mRNA expression of the p53 isoforms strongly differed between clearCa-3 and clearCa-6 although both cell lines revealed a similar induction of p53 target genes. Nevertheless, differential regulation of the isoform expression pattern upon treatment with genotoxic substances could contribute to the similar results on induction of p53 inducible genes in the two cell lines. Thus, changes of the p53 isoform expression pattern upon treatment with Topotecan in both cell lines were investigated. In clearCa-6 the relative mRNA expression of the isoforms p53, p53beta and p53gamma was significantly increased after 12 h Topotecan treatment when compared to untreated cells. No significant alteration of the expression level of all del133p53 isoforms could be detected in this cell line and only del40p53 was significantly upregulated, whereas del40p53beta and del40p53gamma mRNA
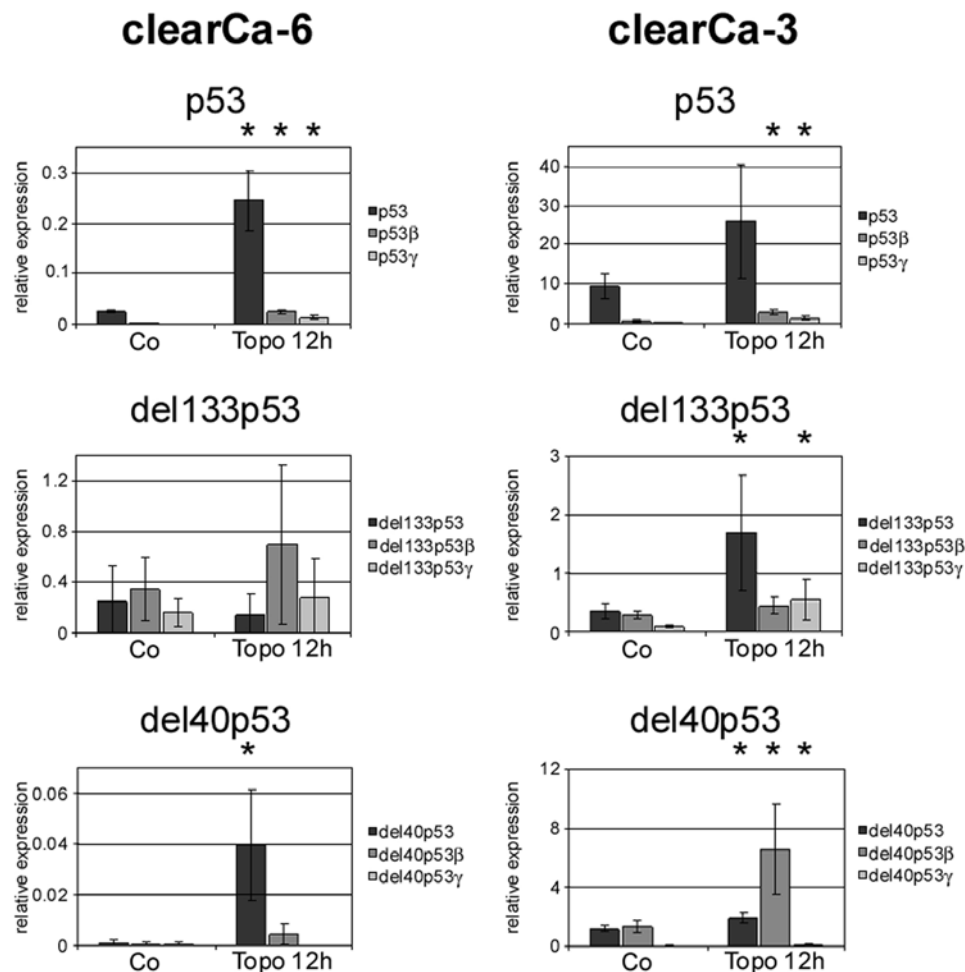
Figure 7. Differential regulation of p53 isoform expression by Topotecan in clearCa-6 and clearCa-3. In clearCa-6 p53, p53beta, p53gamma and del40p53 were significantly upregulated after Topotecan treatment for 12 h at a concentration of 1 microgram/ml (Topo 12 h), whereas no regulation of the other isoforms could be detected. In clearCa-3, p53beta, p53gamma, del133p53, del133p53gamma and all del40p53 isoforms were significantly upregulated after Topotecan treatment for 12 h at a concentration of 1 microgram/ml (Topo 12 h), whereas no regulation of p53 and del133p53beta could be detected. (Shown are the mean and standard deviations of three independent experiments.)

expression remained unchanged (Figure 7). These results could be confirmed at the protein level for p53 by Western blot (data not shown).

In contrast, in clearCa-3 only p53beta and p53gamma but not p53 were significantly induced by Topotecan after 12 h. Furthermore, the inhibitory isoforms del133p53 and del133p53gamma, as well as all del40p53 isoforms were significantly upregulated after Topotecan treatment (Figure 7). These results could mostly be confirmed at the protein level for p53 by Western blot (data not shown).

Thus, clearCa-6 and clearCa-3 strongly differed in the transcriptional regulation of the p53 isoforms, although the p53 target genes investigated were induced to a similar extent.

5. DISCUSSION

RCCs reveal a functional loss of p53 contributing to the marked resistance towards conventional chemotherapy. However, the exact cellular mechanisms responsible for this functional loss are not clear. One of these mechanisms could be the regulation of p53 function by differential regulation of its isoforms harbouring either activating or inhibiting effects on p53 target gene expression. Since deregulation of p53 isoform expression could already be shown for melanomas, OLP, ovarian cancer, AML and SCCHN (14-18), this study addressed the question whether p53 isoform expression changes during carcinogenesis and progression of RCCs *in vivo*. Furthermore, p53 isoform expression and regulation as well as induction of p53 dependent target genes and induction of apoptosis were compared in RCC cell lines with similar sensitivities towards Topotecan-induced cell death. The

aims of this study were 1) to analyse the differential expression of all p53 isoforms throughout initiation and progression of RCCs *in vivo*, 2) to examine their differential regulation by chemotherapy *in vitro* and 3) to correlate p53 isoform expression and regulation to the induction of so-called "p53-target genes", sensitivity towards chemotherapy and induction of apoptosis.

Therefore, we examined the expression of the nine p53 isoforms, p53, p53beta, p53gamma, del40p53, del40p53beta, del40p53gamma and del133p53, del133p53beta as well as del133p53gamma for the first time in RCCs by qPCR. We did not investigate deltap53 expression, since the existence of this isoform is discussed controversially in the literature. Although its existence could obviously be shown in breast cancer (22), a study of Murray-Zmijewski and coworkers (23) was not able to demonstrate its expression in a large number of either non-neoplastic tissues of different origins or tumor tissues. Thus it is not clear whether deltap53 is just an artefact evolving from reverse transcription (11).

In the study presented here, p53 isoform mRNA expression was weak in all non-neoplastic tissues. Interestingly, during carcinogenesis and tumor progression the expression of nearly all isoforms significantly changed: The expression of the transcription activating isoforms p53, p53beta and p53gamma was significantly increased in RCCs of the pT1 and pT2 stages compared to non-neoplastic tissues. While p53beta and p53gamma expression returned to the level of non-neoplastic renal tissue in pT3 carcinomas, the expression level of p53 remained increased in pT3 carcinomas. This was surprising to some extent, since one would expect a shift towards a more p53-inhibiting isoform pattern during carcinogenesis and tumor progression. However, these observations are consistent with the findings in breast cancer, OLP and SCCHN, which could also demonstrate an overexpression of these p53 isoforms (11, 15, 18).

The increase in the activating p53 isoforms was paralleled by a decrease of the p53-inhibitory del133p53 isoforms, including del133p53beta and del133p53gamma, in early tumor stages (pT1 and pT2), whereas del133p53 expression remained relatively constant during carcinogenesis and tumor progression. Only during further tumor progression to pT3 RCCs could a significant increase in del133p53 and del133p53beta be demonstrated compared to pT1 and pT2 tumors, respectively. However, there was no difference in the expression levels of all del133p53 isoforms in pT3 tumors and non-neoplastic renal tissues. These results are in contrast to the observations made in breast cancer, OLP and SCCHN, which could show an increased expression of the del133p53 isoforms in these diseases. This suggests a different function of p53 isoform expression in RCCs (11, 15, 18).

Studies regarding the del40p53 isoform expression in cancers *in vivo* are sparse. In our study, a significant increase of two of the del40p53 isoforms, del40p53 and del40p53gamma, could be demonstrated in pT2 and pT3 RCCs, whereas no significant differences in

mRNA expression levels could be demonstrated comparing non-neoplastic renal tissues and pT1 carcinomas. Thus, the del40p53 isoforms showed a differential regulation especially during tumor progression. Interpretation of these results, however, is challenging since the function of these isoforms has not been completely elucidated, yet: The del40p53 isoforms themselves do not possess transcriptional activation capacity but modulate the p53 activity on different target genes. Furthermore, del40p53 can prevent p53 from degradation by Mdm2 (13). As a result, their function as activators or inhibitors of p53 dependent gene induction depends on the ratio of del40p53 to p53, on the target gene (12) and possibly also on the tissue examined.

Taken together, *in vivo* renal cell carcinomas revealed a shift towards a more p53 activating isoform expression pattern during tumor initiation and tumor progression. The observed increase in inhibitory del133p53 isoforms during tumor progression did not exceed the expression in non-neoplastic renal tissue. Furthermore, expression of del40p53beta and del40p53gamma could be demonstrated for the first time in this study: these isoforms were regarded as theoretically possible but not actually existing splice-variants (11). Expression of del40p53 and del40p53gamma was significantly increased in late tumor stages. The meaning of these results for the transcriptional activity of p53, however, is currently difficult to assess.

In vitro analysis of two RCC cell lines with similar sensitivities towards Topotecan induced cell death revealed a similar induction of important p53 target genes. However, they strongly differed in the induction of apoptosis: clearCa-6 and clearCa-3 both revealed a significant induction of p21, Noxa and PIDD. In contrast, Bax, another very important proapoptotic gene, was not induced in either cell line, a fact that might contribute to apoptosis resistance in RCCs. However, clearCa-6 revealed a strong induction of apoptosis as determined by PARP-cleavage and caspase-2-, -3/-7-, -8- and -9-like activities, whereas clearCa-3 revealed only weak PARP-cleavage and a weak activation of only caspase-2-like activities. The other caspases were not significantly induced. Obviously the induction of p53 dependent genes did not predict the sensitivity towards Topotecan-induced apoptosis in these cell lines, pointing to the fact that maybe transcription factors other than p53 or cellular mechanisms other than p53 gene induction - probably located downstream of p53 gene induction - seem to be important for apoptosis induction in these RCC cell lines (6, 24, 25).

Furthermore, although revealing similar induction of p53 inducible genes, both cell lines exhibited a very different basal mRNA expression pattern of the p53 isoforms. Expression of p53, p53beta and p53gamma was approximately 400 times higher and the expression of del40p53, del40p53beta and del40p53gamma was approximately 1000 times higher in clearCa-3 than in clearCa-6. Untreated clearCa-6 was more or less devoid of transcription activating p53 isoforms, pointing to the fact that obviously other transcription factors than p53 were responsible for the induction of the so-called "p53-inducible genes". Only the del133p53 isoforms were

expressed at a similar level. Additionally, the p53 isoforms were strongly differentially regulated in the cell lines upon treatment with Topotecan: In clearCa-6 the transcription activating isoforms p53, p53beta and p53gamma as well as del40p53 were significantly induced, with the expression of the other isoforms remaining unchanged. In clearCa-3, however, p53 expression did not significantly change and only p53beta and p53gamma were increased. Furthermore, the transcription inhibiting del133p53 and del133p53gamma as well as all del40p53 isoforms were induced.

As a result, the sensitivity of RCC cell lines towards p53-induced gene induction could not be predicted from the expression pattern of the p53 isoforms in untreated cells as well as changes in the expression pattern upon treatment with a drug simulating DNA-damage. This confirms our *in vivo* results that the well known strong therapy-resistance of RCCs paradoxically correlates with an upregulation of the transcription activating p53 isoforms and downregulation of the inhibitory p53 isoforms.

Our study makes it plausible that analysis of the p53 isoform mRNA expression pattern alone is not sufficient to draw conclusions on the functional activity of p53 in RCCs. Moreover, the emerging network of p53 transcriptional and functional regulations seems to play the decisive role: It is well known that the function, expression and stabilization of p53 protein depend on multiple regulatory mechanisms. The interaction of p53 with Mdm2 results in ubiquitinylation and degradation of p53, thus leading to a short half-life of the p53 protein of just 20 minutes (12, 26, 27). The N-terminal deleted isoforms do not harbour a Mdm2 binding site, which increases the half-life. The half-life of del40p53, for example, can be increased to 9.5 h (28), further complicating the interpretation of the results on the mRNA level. Furthermore, posttranslational modifications such as phosphorylation by ATM/ATR or the effector kinases Chk1 and Chk2, acetylation by means of CBP/p300 (3, 29) as well as methylation by CARM1 and PRMT1 (29, 30) and sumoylation by SUMO-1 stabilize p53 and thereby enhance p53-dependent gene induction (31, 32). Cofactors, including coactivators (JMY and STRAP-1) as well as coinhibitors (iASPP, mSin3A and Daxx) further modulate the transcriptional function of p53 (3, 33-36). Finally the function of p53 can at least in part be replaced by p63 and p73 (7, 11). p53 protein stabilization seems to be of particular importance in regulating p53 function depending on, for instance, Mdm2, HIPK2, HAUSP (37-39).

As a result, our study shows for the first time the differential expression of all p53 isoforms in a tumor *in vivo* using the example of RCCs. We also demonstrate differential regulation of p53 isoforms throughout tumor initiation and progression in RCCs with a shift of p53 isoform expression towards those favoring transcription activation. Furthermore, our *in vitro* studies are the first to reveal that p53 isoforms are strongly differentially regulated by chemotherapy in RCC cell lines, which might also be important for the therapy of other tumors. Therefore, expression and regulation of so-called "p53-

target genes" were obviously at least in part regulated by other transcription factors. In addition, we provide novel evidence that p53 isoform expression in RCC cell lines is – probably in contrast to other tumors - of minor importance for sensitivity towards chemotherapy and induction of apoptosis.

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Abbreviations: AML: acute myeloid leukemia, Arf: ADP-ribosylation factor, ATM: ataxia telangiectasia mutated, ATR: ataxia telangiectasia and Rad3 related, CARM1: coactivator-associated arginine methyltransferase 1, CBP: CREB binding protein, HAUSP: Herpesvirus-associated ubiquitin-specific protease, HIPK2: homeodomain interacting protein kinase 2, iASPP: inhibitor of ASPP protein, JMY: junction mediating and regulatory protein, Mdm2: murine double minute 2, MdmX: murine double minute X, OLP: oral lichen planus, PARP: poly (ADP-ribose) polymerase, *PIDD:* p53-induced protein with a death domain, PRMT1: protein arginine methyltransferase 1, RCC: renal cell carcinoma, SCCHN: squamous cell carcinoma of the head and neck, SDHA: succinate dehydrogenase complex, subunit A, STRAP-1: serine/threonine kinase receptor associated protein 1, SUMO-1: Small ubiquitin-like protein 1

Key Words: Renal Cell Carcinoma, p53, p53 isoforms, Topotecan, Apoptosis

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