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Topoisomerase II α Status in Renal Medullary Carcinoma: Immuno-Expression and Gene Copy Alterations of a Potential Target of Therapy

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Abstract

Purpose—Renal medullary carcinoma is an aggressive renal neoplasm without currently available effective therapy to our knowledge. Topoisomerase II α is a gyrase involved in cell proliferation, and DNA maintenance and repair. Topoisomerase II α is a target of inhibiting agents such as anthracyclines. Triggered by a recent response to topoisomerase II α inhibitors in a patient with renal medullary carcinoma, we evaluated topoisomerase II α expression in relation to the proliferation index and topoisomerase II α gene copy number status in a larger series of patients with renal medullary carcinoma.

Materials and Methods—Archival tissues from 14 renal medullary carcinomas were retrieved from our 3 institutions. Immunohistochemistry was performed using monoclonal antibodies for topoisomerase II α and Ki67. The percent of cells with positive nuclear staining was assessed in the highest area of expression for each marker. A previously suggested greater than 5% cutoff was used for topoisomerase II α over expression. The topoisomerase II α gene copy number was evaluated using fluorescence in situ hybridization. Locus specific topoisomerase II α gene and chromosome 17 centromere probes were used. The total number of topoisomerase II α and chromosome 17 centromere signals was counted in 150 cells per tumor and a topoisomerase II α -to-chromosome 17 centromere signal ratio was calculated in each tumor. A topoisomerase II α -to-chromosome 17 centromere ratio of 2.0 or greater and less than 0.8 was used as a cutoff for amplification and deletion, respectively. The percent of tumor cells with polysomic, eusomic or monosomic chromosome 17 status was also determined.

Results—On immuno-expression analysis topoisomerase II α immunohistochemistry was technically inconclusive in 1 renal medullary carcinoma. Topoisomerase II α was over expressed in 11 of 13 renal medullary carcinomas (85%) (median 50%, range 1% to 80%). As expected, a high Ki67 proliferation index was noted in 13 of 14 tumors (median 87.5%, range 2% to 100%). Ki67 expression was greater than topoisomerase II α expression in all 13 informative tumors. A strong, statistically significant correlation was found for topoisomerase II α and Ki67 expression (pairwise CC 0.9, $p = 0.0000$). Topoisomerase II α over expression was associated with shorter survival ($p = 0.000$). On fluorescence in situ hybridization no topoisomerase II α amplification was detected in any of the 14 renal medullary carcinomas, including the 11 with topoisomerase II

α over expression. Topoisomerase II α gene deletions were noted in 4 tumors. Two of 4 deletions were associated with chromosome 17 monosomy and 2 were in eusomic chromosome 17 tumors.

Conclusions—Topoisomerase II α is over expressed in 85% of renal medullary carcinomas, potentially supporting the use of topoisomerase II α inhibitor agents to treat this aggressive renal tumor. Our findings suggest that topoisomerase II α over expression in our renal medullary carcinoma cohort was not due to gene amplification, but rather to transcriptional or post-transcriptional modifications. The significance of the incidentally found topoisomerase II α deletions in 28% of renal medullary carcinomas requires further evaluation.

Keywords

kidney; carcinoma; kidney medulla; DNA topoisomerase II alpha; gene deletion

Renal medullary carcinoma is a rare renal malignancy that primarily affects young patients with sickle cell hemoglobinopathy. RMC is a highly aggressive tumor with a dismal reported average survival of 15 weeks.^{1–6} Therefore, finding effective therapeutic strategies for RMC, including the identification of molecular targets, is crucial.

TOP2A is a nuclear enzyme that controls DNA topological structure and cell cycle progression.⁷ Recently using cDNA microarray analysis our group identified TOP2A as 1 of the top 40 over expressed genes in RMC.⁸ Furthermore, we achieved an initial complete response and 24-month overall survival after administering the TOP2A inhibitor chemotherapeutic agent doxorubicin in 1 patient with RMC. Triggered by that encouraging response and given the prior demonstration of a therapeutically predictive role for TOP2A gene status in other solid tumor cases,⁹ we evaluated TOP2A gene expression and copy number status in a series of primary and metastatic RMCs.

MATERIALS AND METHODS

Patient Population

Material from a total of 14 primary and metastatic RMCs were obtained from the surgical pathology file at 3 institutions, including the consultation file of one of us. Seven cases were retrieved from the surgical pathology files at Johns Hopkins University, Baltimore, Maryland; 5 that were the subject of a prior clinicopathological report of RMC⁶ were obtained from the department of pathology, State University of Campinas, Sao Paulo, Brazil; and the remaining 2 were retrieved from the surgical pathology archives, Wake Forest University, Winston-Salem, North Carolina.

All available hematoxylin and eosin stained slides were reviewed by 2 pathologists and the initial diagnosis of RMC was confirmed. In each case a representative block was selected for IHC analysis and FISH. Three consecutive unstained sections were used for TOP2A, Ki67 and FISH analysis.

IHC of TOP2A Expression and Ki67 Proliferation Index

TOP2A—Immunostains were performed using standard immunohistochemistry procedure. Briefly, unstained sections were deparaffinized, rehydrated in graded alcohols and subjected to heat induced antigen retrieval for 60 minutes in ethylenediaminetetraacetic acid, pH 9.0, using a microwave oven. Section were then incubated for 40 minutes at 37C with TOP2A monoclonal antibody (CNL clone, Novocastra, New Castle, United Kingdom) (1:100). After applying secondary antibody the slides were developed for 5 minutes using 3-3'-diaminobenzidine chromogen and counterstained with hematoxylin. Appropriate negative and positive controls were used. TOP2A expression was assessed as the percent of cells with

TOP2A positive nuclear staining in the highest expression area. A previously suggested greater than 5% over expression cutoff¹⁰ was used to indicate TOP2A over expression.

Ki67—Ki67 immunostaining was performed using a similar protocol with Ki67 primary antibody (k-2 clone, Ventana, Phoenix, Arizona) (1:1,000) using a 30-minute incubation time. The percent of tumor cells with positive nuclear staining for Ki67 in the highest area was used as the Ki67 proliferation index.

TOP2A/CEP17 Interphase FISH

LSI® TOP2A SpectrumOrange™/CEP17 SpectrumGreen™ labeled FISH probes were used. Paraffin embedded tissue sections (4 μ) were baked at 56C for 2 hours, deparaffinized and rehydrated with xylene and graded ethanol. Tissue sections were pretreated using a Paraffin Pretreatment Reagent Kit III, (Abbott Molecular, Des Plaines, Illinois). Briefly, sections were immersed in 45% formic acid/0.3% hydrogen peroxide for 15 minutes, washed and pretreated at 80C with pretreatment solution composed of 1 M sodium thiocyanate/1 M tris base (pH 8.0) for 10 minutes and digested with proteinase K at 37C for 5 minutes. Tissue sections and FISH probes were co-denatured at 94C for 5 minutes and hybridized overnight at 37C in a ThermoBrite® humid chamber. Post-hybridization washing was done with 2 × saline sodium citrate/NP-40 for 5 minutes at room temperature and then for 2 minutes at 73C. Slides were counterstained with 4'-6-diamidino-2-phenylindole. Slides were imaged using an Axioskop™ epifluorescence microscope. Fluorescent images were captured with a cooled charge coupled device MicroMAX digital camera (Princeton Instruments, Trenton, New Jersey).

TOP2A FISH Interpretation

A total of 150 nuclei per tumor were scored for TOP2A and CEP17 signals. Only tumor cells with nonoverlapping nuclei were scored. The total number of TOP2A and CEP17 signals in 150 nuclei was first recorded for each RMC. The TOP2A-to-CEP17 signal ratio was then calculated in each tumor. According to criteria suggested by Hicks et al¹¹ a tumor was categorized as TOP2A amplified when the TOP2A-to-CEP17 ratio was 2.0 or greater, TOP2A nonamplified when the ratio was between 0.8 and 1.9, and TOP2A deleted when the ratio was less than 0.8.

Each tumor was also classified by Ch17 copy number status (polysomic, eusomic or monosomic for Ch17) based on the scheme of Mueller et al.¹² RMCs with greater than 50% of cells with greater than 2 CEP17 signals were categorized as polysomic, those with more than 50% of cells with 1 CEP17 signal were categorized as monosomic and those with more than 50% of cells with 2 CEP17 signals were categorized as eusomic for Ch17.

Statistical Analysis

Findings were analyzed using Stata™ 9.2 software. Equality of population medians among groups was tested using the Kruskal-Wallis test for nonparametric 1-way ANOVA by ranks. Pairwise correlation coefficients were calculated to test relationships among variables.

RESULTS

Patient Demographics and Clinical Data

Table 1 lists patient demographics and clinical data. Patients were 8 to 33 years old (mean age 20). Of the patients 12 were male and gender was not available in the remainder. A striking right side predilection was noted with a right kidney primary tumor in 10 of 11 cases. Ethnicity, which was known in 9 patients, was black in 5, African Brazilian in 3 and

white in 1. Sickle cell hemoglobinopathy was documented in 11 patients. Hemoglobin status was not available in the remainder. One or more of the classic renal tumor triad was the presenting sign/symptom in 11 patients. While in 2 patients metastasis was the first manifestation of disease, widespread metastasis manifested shortly after diagnosis in an additional 6 patients.

Followup data were available on 9 patients. Overall survival was 3 weeks to 8 years (mean 17.6 months). Treatment approaches varied widely among patients with only limited success. Six of our patients received chemotherapeutic agents. In the 3 patients with a treatment regimen containing TOP2A inhibitor agents 4, 9 and 23 months of survival (mean 12) were achieved, respectively. In the 3 remaining patients who received agents not known to target TOP2A (combination gemcitabine with cisplatin or paclitaxel) survival was 0.75, 7 and 15 months (mean 7.6).

Immunohistochemistry

Table 2 lists immunostaining and FISH results. For TOP2A IHC was technically inconclusive in 1 RMC. The incidence of tumor cells with positive TOP2A nuclear staining was 1% to 80% (median 50%, mean 46.5%). Using a 5% cutoff TOP2A over expression was present in 11 of 13 RMCs. For Ki67 the tumor proliferation index was 2% to 100% (median 87.5%, mean 73%). The Ki67 proliferation index was higher than the percent of TOP2A nuclear positivity in all 13 informative tumors. The latter was reflected by Ki67 staining not only in cells expressing TOP2A, but also in cells negative for TOP2A on stained serial sections (fig. 1). A strong, statistically significant correlation was noted between TOP2A and Ki67 expression in RMCs (pairwise CC 0.9, $p = 0.0000$).

FISH

Based on TOP2A-to-CEP17 signal ratio calculations none of our 14 RMCs were TOP2A amplified. Four RMCs showed evidence of TOP2A gene deletion (TOP2A-to-CEP17 ratio less than 0.8). None of the 14 examined RMCs had evidence of Ch17 polysomy. Of the tumors 12 (85.7%) were eusomic, including 2 of the 4 with TOP2A deletions. The remaining 2 RMCs (14.2%) were monosomic for Ch17, resulting in TOP2A deletions (table 3 and fig. 2).

TOP2A Over Expression and Gene Copy Number

TOP2A over expression was present in all 10 RMCs with nonamplified TOP2A gene copy number status. On the other hand, only 1 of 3 RMCs with TOP2A gene deletion on FISH had evidence of TOP2A over expression on IHC. The remaining 2 RMCs with TOP2A gene deletion on FISH were the only 2 without evidence of TOP2A over expression in the entire cohort. When the relationship of the percent of sub-population of cells showing a TOP2A-to-CEP17 ratio of greater than 1.9 to the proportion expressing TOP2A in a given RMC was analyzed, a statistically nonsignificant trend toward positive correlation was noted on pairwise analysis (pairwise CC 0.33, p not significant).

Survival, TOP2A Expression and Gene Copy Number, and Ki67

TOP2A over expression was associated with shorter survival in patients with over and nonover expression (mean 8.9 vs 48 months, $p = 0.000$). No statistically significant relationship was present between TOP2A gene copy number status and survival (p not significant). The latter was also true for the relationship of Ki67 expression and survival (p not significant).

DISCUSSION

During DNA replication TOP2A catalyzes the adenosine triphosphate dependent transport of 1 intact DNA double helix through another as well as chromosomal segregation.⁷ Higher TOP2A expression strongly correlates with tumor proliferation in breast, gastric and colon cancer.^{13,14} TOP2A is a molecular target for several anticancer agents, eg anthracycline, by increasing the concentration of covalent TOP2A enzyme-DNA complexes and ultimately triggering the cell death pathway.¹⁵ Prior studies demonstrated a correlation between TOP2A protein expression and the clinical response to anti-TOP2A agents in cases of breast carcinoma¹⁶ and in tumor cell lines, including renal cell carcinoma.^{17,18}

Using gene expression profiling our group previously observed TOP2A gene mRNA over expression⁸ in 2 RMCs. In the current study we expanded our prior observation by analyzing the range of TOP2A protein expression on IHC in 14 RMCs. TOP2A was over expressed in 11 of the 13 informative RMCs. Our finding could be viewed as additional rationale for using TOP2A targeting chemotherapeutic agents for this rare aggressive renal malignancy.

By simultaneously assessing the Ki67 proliferation index and TOP2A expression in sequential serial RMC sections we confirmed the expected correlation between the 2 proliferation associated markers with generally higher Ki67 expression. However, it is of interest that, although a subset of cells in a given tumor co-expressed TOP2A and Ki67, the expression pattern was not completely overlapping. Many Ki67 positive tumor cells lacked TOP2A expression. Similarly tumor cells expressing TOP2A that were negative for Ki67 were also observed, although less frequently. The latter pattern suggests other than proliferation related TOP2A expression in this mitotically active group of high grade renal neoplasms. Future studies using dual color immunostaining would further characterize the previous finding.

Our finding of a lack of correlation between TOP2A expression and gene copy amplification indicates that potential mechanisms other than gene amplification are responsible for TOP2A over expression in RMC. The latter may include transcriptional or post-translational modifications of such a strictly regulated enzyme.¹⁹ Other factors could include elongation of TOP2A enzyme nuclear half-life or a decreased rate of its degradation.²⁰ Interestingly Tretiakova et al also noted the lack of correlation between TOP2A expression and gene amplification in cases of Wilms tumor, another aggressive type of renal malignancy.²¹ In this regard in 4 of 10 TOP2A over expressing RMCs we observed a subpopulation of 20% to 30% of tumor cells in which the TOP2A-to-CEP17 signal ratio exceeded the 1.9 amplification cutoff (table 2). Although these 4 tumors were categorized as TOP2A gene nonamplified based on their overall TOP2A-to-CEP17 signal ratio, gene copy amplification in a subpopulation of tumor cells could have accounted for TOP2A over expression in such tumors. Although a weak correlation was found between the percent of the subpopulation of cells showing a TOP2A-to-CEP17 ratio of greater than 1.9 and the proportion expressing TOP2A in a given RMC, the trend was not statistically significant. Performing combined TOP2A immunofluorescence/TOP2A FISH would be needed to better identify such a potential association.

In our cohort TOP2A over expression but not TOP2A gene copy number status or Ki67 expression was associated with shorter survival. However, findings require further confirmation, given the small number of cases lacking TOP2A over expression in the current study.

To our knowledge this is the first study to show evidence of TOP2A gene deletion in RMCs. Four of 14 tumors (29%) showed TOP2A gene allelic loss on FISH. Two of the 4 deletions were associated with Ch17 monosomy and the remaining 2 tumors were eusomic for Ch17.

Furthermore, the only 2 RMCs in our cohort without evidence of TOP2A protein over expression (less than a 5% TOP2A expression rate) had evidence of TOP2A gene deletion with 1 eusomic and 1 monosomic for Ch17. It is possible but speculative that low protein expression in the latter 2 RMCs was related to the associated gene loss.

Our finding was that 6 of 10 RMCs with TOP2A over expression finally received chemotherapy. Although average survival in the 3 patients who received TOP2A inhibitor agents was slightly higher than that in those who received other agents (12 vs 7.6 months), a definite conclusion could not be drawn from our limited number of patients. Furthermore, the implications of the low expression and gene copy loss in regard to predicting the response to TOP2A inhibitor agents could not be determined in our study since no patient with a TOP2A deleted tumor received TOP2A inhibitor agents.

CONCLUSIONS

We noted TOP2A over expression in most RMCs in our relatively large cohort of this rare tumor type. Our finding potentially lends support to the use of TOP2A inhibitors to treat this aggressive renal tumor. Prospective studies, preferably in a larger patient cohort, are needed to properly define the potential role of TOP2A evaluation for predicting treatment response. Our study also suggests that TOP2A over expression in RMC cannot be attributable to TOP2A gene amplification, which was absent. On the other hand, TOP2A deletion and Ch17 monosomy were noted in an RMC subset, which are findings that require further validation and analysis.

Acknowledgments

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Abbreviations and Acronyms

CEP17	Ch17 centromere
Ch17	chromosome 17
FISH	fluorescence in situ hybridization
IHC	immunohistochemistry
RMC	renal medullary carcinoma
TOP2A	topoisomerase II α

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Figure 1. RMC histological features. *A*, intratumoral sickled red blood cells. *B*, IHC shows high proliferation index for Ki67. *C*, TOP2A over expression. *B* and *C*, note incomplete Ki67 and TOP2A staining overlap in 2 consecutive tumor sections. H & E, reduced from $\times 200$.

Figure 2.

Dual color FISH for TOP2A gene in RMC. *A*, nonamplified TOP2A. Gene tumor nuclei show 2 orange (TOP2A gene copy) and 2 green (Ch17 centromeric region) signals per nucleus. *B*, TOP2A gene copy deletion. Several tumor nuclei show 1 or 2 green signals per nucleus but lack TOP2A orange signal compared to cell with 2 green and 2 orange signals at mid left. *C*, TOP2A nonamplified tumor shows focal TOP2A amplification in tumor cell subpopulation with 4 or more orange signals in some cells associated with 2 green signals. Overall tumor categorization was TOP2A nonamplified due to overall low TOP2A-to-CEP17 ratio. Reduced from $\times 1,000$.

Table 1

Demographics and physical features in 14 patients with RMC

Pt No.—Age—Sex	Ethnicity	Hemoglobin Status	Side	Presentation	Treatment	Chemotherapy	Survival
1—17—M	Black	Sickle cell trait	Rt	Renal mass at cardiac malformation followup	Radical nephrectomy + chemotherapy	Gemcitabine, cisplatinum + taxol	15 Mos
2—30—M	Black	Sickle cell trait	Rt	Renal mass, hematuria, flank pain	Radical nephrectomy + chemotherapy	Gemcitabine + cisplatinum	7 Mos
3—27—M	Not available	Not available	Rt	Not available	None	Not available	Not available
4—11—M	Not available	Sickle cell trait	Not available	Lymph node metastasis	Radical nephrectomy	Not available	Not available
5	Not available	Sickle cell trait	Not available	Not available	Radical nephrectomy	Not available	Not available
6—33—M	Black	Sickle cell trait	Rt	Abdominal pain	Radical nephrectomy + chemotherapy	Etoposide	23 Mos
7—25—M	Black Brazilian	Sickle cell trait	Rt	Hematuria	Radical nephrectomy + chemotherapy	Metothrexate, vinblastin, doxorubicin + cisplatinum	4 Mos
8—11—M	Black Brazilian	Sickle cell trait	Rt	Hematuria	Radical nephrectomy + chemotherapy	Etoposide, doxorubicin, cyclophosphamide + vincristine	9 Mos
9—14—M	White	Not available	Rt	Hematuria, flank pain, wt loss	None	None	4 Days
10—13—M	Not available	Sickle cell trait	Rt	Hematuria dyspnea	Not available	Not available	3.75 Mos
11—8—M	Black Brazilian	Sickle cell trait	Rt	Hematuria, pain	Radical nephrectomy	None	96 Mos
12	Not available	Not available	Not available	Not available	Radical nephrectomy	Not available	Not available
13—24—M	Black	Sickle cell trait	Rt	Renal mass, hematuria, flank pain	None	Not available	Not available
14—26—M	Black	Sickle cell trait	Lt	Lung metastasis	Chemotherapy	Pacitaxel, gemcitabine + cisplatinum	3 Wks

Table 2
TOP2A and Ki67 on IHC in 14 patients with RMC and associated TOP2A gene status on FISH

Pt. No.	IHC			FISH		
	% Ki67 Pos Cells	% TOP2A Pos Cells	TOP2A Protein Over Expression	% Cells TOP2A/CEP17 2.0 or Greater	TOP2A Gene Status	
1	90	50	Yes	30	Nonamplified	
2	90	70	Yes	23	Nonamplified	
3	100	80	Yes	11	Nonamplified	
4	70	30	Yes	6	Nonamplified	
5	100	60	Yes	3	Nonamplified	
6	75	40	Yes	20	Nonamplified	
7	85	40	Yes	21	Nonamplified	
8	90	50	Yes	9	Nonamplified	
9	2	1	No	4	Deleted	
10	90	70	Yes	7	Nonamplified	
11	20	4	No	3	Deleted	
12	35	Inconclusive	Inconclusive	0	Deleted	
13	95	70	Yes	15	Nonamplified	
14	80	40	Yes	0	Deleted	

Table 3

TOP2A gene copy status according to Ch17 copy number in 14 RMCs

Ch17 Status	No. TOP2A Gene Copy Status			Total No.
	Nonamplified	Deleted	Amplified	
Monosomy	0	2	0	2
Eusomy	10	2	0	12
Polysomy	<u>0</u>	<u>0</u>	<u>0</u>	<u>0</u>
Totals	10	4	0	14