Intragenic *PTEN/MMAC1* Loss of Heterozygosity in Conventional (Clear-Cell) Renal Cell Carcinoma is Associated with Poor Patient Prognosis

Marija Velickovic, B.Sc., Brett Delahunt, M.D., Bryan McIver, M.B.B.S., Ph.D., Stefan K. G. Grebe, M.D.

Department of Pathology and Molecular Medicine (MV, BD, SKGG), Wellington School of Medicine and Health Sciences, Wellington, New Zealand; and Department of Medicine (BM), Mayo Foundation and Clinic, Rochester, Minnesota

Inactivation of the *PTEN/MMAC1* tumor suppressor gene has been linked to tumor progression in several human malignancies. However, the role of *PTEN/MMAC1* in the development and progression of the major renal cell carcinoma morphotypes remains controversial. We examined microdissected specimens from 80 conventional (clear cell) renal cell carcinomas (cRCC), 27 papillary renal cell carcinomas (pRCC), and 16 chromophobe renal cell carcinomas (chRCC) for loss of heterozygosity (LOH) at and around the *PTEN/MMAC1* locus and for mutations in the *PTEN/MMAC1* gene. The results of the molecular studies were correlated with tumor stage, grade, and patient survival. LOH at one or more of the examined loci occurred in 37.5% of cRCC, 29.6% of pRCC and 87.5% of chRCC specimens. The chRCC specimens showed increasing rates of LOH the further that a marker was located toward the q telomer of chromosome 10, consistent with nonspecific genetic disarray in genomically highly unstable tumors. No such pattern was discernible in the cRCC and pRCC. In the cRCC, LOH at intragenic *PTEN/MMAC1* microsatellite markers (indicating deletional events involving the actual *PTEN/MMAC1* gene) was significantly associated with tumor death, with 85.7% of such patients dying, whereas only 45.3% of patients without intragenic LOH died ($P = .018$). There were no *PTEN/MMAC1* mutations in our specimens. We conclude that *PTEN/MMAC1* inactivation may play a role in the progression of cRCC. Biallelic inactivation may

preferentially occur by nonmutational mechanisms, or, alternatively, haploinsufficiency of *PTEN/MMAC1* may be sufficient to affect tumor progression in cRCC.

KEY WORDS: Loss of heterozygosity, Mutation screening, *PTEN/MMAC1*, Renal cell carcinoma, Tumor survival.

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As a group, renal epithelial malignancies are frequently encountered in clinical practice. It is now appreciated that rather than being a single tumor type, they consist of a variety of morphotypes that show differing clinical behavior (1–3). The most frequently encountered subtype is conventional (clear cell) renal cell carcinoma (cRCC), representing 70% of all adult malignant primary renal tumors, whereas papillary renal cell carcinoma (pRCC) account for 10–15%, and chromophobe renal cell carcinoma (chRCC), for approximately 5%. The remainder is made up of collecting-duct carcinomas and unclassifiable tumors (2–5).

Several morphotype-specific somatic genetic changes have been identified in renal cell carcinoma (4). Inactivation of the tumor suppressor gene *VHL* has been established as an early step in the pathogenesis of cRCC, whereas pRCC has been reported to show trisomy 7 or 17, loss of the Y chromosome, and, in familial cases, mutations in the *c-MET* oncogene at 7q31 (4–7). Finally, chRCC is characterized by multiple, large chromosomal deletions, involving chromosomes 1, 2, 6, 10, 13, and 17 (8–10). Although these somatic genetic changes provide some insights into the molecular pathogenesis of renal cell tumors and may even aid in the molecular differential diagnosis of renal cell carcinoma morphotypes, they have not, to date, been linked to tumor progression or prognosis. The identification of somatic genetic changes that correlate with prognosis in some or all of the RCC

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Address reprint requests to: Stefan K. G. Grebe, M.D., Department of Pathology & Molecular Medicine, Wellington School of Medicine and Health Sciences, Mein Street, Newtown, PO Box 7343, Wellington South, Wellington, New Zealand; e-mail: grebs@wnmeds.ac.nz; fax: 64-4-389-5725.

morphotypes would not only aid in the understanding of RCC pathogenesis but might also be of significant clinical value.

In many human malignancies, inactivation of the *PTEN/MMAC1* tumor suppressor gene is associated with tumor progression and adverse patient outcome (11, 12). This gene maps to 10q23, a region of frequent genetic deletions in chRCC and, to a lesser degree, in cRCC. Because inactivation of tumor suppressor genes most commonly involves deletion of one of the gene's alleles, this raises the possibility that *PTEN/MMAC1* may be inactivated in some RCC. Initial screens of 9 RCCs seemed to support this hypothesis, showing loss of heterozygosity (LOH) in 40% of tumors and mutations in 15% of tumors (11, 12). However, two subsequent larger studies failed to detect any mutations (13, 14). Recently, Alimov *et al.* (15) reported a 34% LOH rate at 10q23–25 but found only three mutations when they screened the subset of tumors that displayed LOH for mutations; Kondo *et al.* (16) detected only five mutations, some of which were heterozygote, in a series of 68 sporadic renal cell carcinomas; and Sukosd *et al.* (17) found high rates of 10q23.3 LOH in chRCC, but only in 2 of 50 cRCC, and detected no *PTEN/MMAC1* mutations in the tumors with LOH.

The published data on the role of *PTEN/MMAC1* inactivation in RCC therefore remain contradictory, and the role of the gene in tumor progression remains uncertain. Furthermore, to date only very limited attempts have been made to correlate molecular findings with clinical data or tumor histopathology or to determine any differences in deletion or mutation rates or patterns between the major RCC morphotypes. We addressed these questions by examining the *PTEN/MMAC1* gene for allelic deletions and mutations in a series of histologically well-characterized cRCC, pRCC, and chRCC. The findings of the molecular studies were then compared between the three morphotypes and correlated with tumor grade, stage, and clinical outcome to determine whether *PTEN/MMAC1* has a role in the progression of the major morphotypes of RCC.

MATERIALS AND METHODS

Tumor Samples

All studies were approved and monitored by the Wellington Ethics Committee. The tumor specimens were obtained from the tissue archives of Wellington Hospital, Wellington, New Zealand and the Klinikum Wuppertal, Wuppertal, Germany. From these sources, we identified 80 cRCC, 27 pRCC, and 16 chRCC from patients for whom a minimum of 5 years of postoperative follow-up data were available. Tumor-specific survival was

recorded for all patients. The sections from each case were reviewed, and the tumors were staged according to the International Union Against Cancer TNM system and graded according to the Fuhrman classification (18, 19). In addition, pRCC were subclassified as Types 1 or 2 on the basis of papillary microarchitecture (5).

DNA Extraction

Paraffin-embedded blocks of formalin-fixed tissue from each case were cut at 10- μ m thickness. Histologically representative, unstained sections from each of the tumors were selected and microdissected into neoplastic components, containing at least 80% tumor cells (typically >90%), and into control components, containing normal renal tissue without any microscopic evidence of tumor. Samples were deparaffinized by successive xylene and ethanol washes and incubated in 150 μ L of DNA extraction buffer (2.7 μ g/ μ L of proteinase K [Roche Diagnostics, Auckland, New Zealand], 100 mM Tris, and 2 mM EDTA, pH 8) at 55° C for 48 hours. Additional proteinase K (2.7 μ g/ μ L) was added after 12 hours. After heat inactivation of proteinase K, aliquots of the digests were used directly for polymerase chain reactions (PCR).

Microsatellite LOH Analysis

LOH analysis was performed by paired normal-tumor microsatellite PCR, using seven markers. Of these, two mapped centromeric to the *PTEN/MMAC1* gene (*D10S579*, *D10S215*), two were located within the gene itself (*AFMa086wg9*, *D10S249I*) and three were localized telomeric to *PTEN/MMAC1* (*D10S608*, *D10S54I*, *AFM280weI*). Figure 1 shows the position of these markers, ordered on the basis of the Genome Database (www.gdb.org/), the Genetic Location Database (http://cedar.genetics.soton.ac.uk/public_html/), the Cooperative Human Linkage Center database (www.chlc.org/), and the dbSTS database (www.ncbi.nlm.nih.gov/).

In a total volume of 25 μ L, the PCR mixtures contained 2.5 μ L of digested sample solution, 200 μ M of deoxynucleotide triphosphates, 500 nM each of forward and reverse primers, 0.02 U/ μ L of *Taq* polymerase, 1 $\times$ PCR buffer, and 1.5 mM Mg²⁺ (all, Roche Diagnostics). One primer was labeled with 6-FAM, HEX, or TET (Applied Biosystems, Melbourne, Australia). For LOH analysis, PCR products were diluted between 1:2 and 1:8, combined with size standard 500-TAMRA (Applied Biosystems) and deionized formamide/loading dye, denatured at 95° C for 3 minutes, quenched on ice, loaded on denaturing 4% polyacrylamide/6 M urea gels, and electrophoresed on an automated sequencer (Model 377; Applied Biosystems).

FIGURE 1. Approximate map positions of the markers used in this study.

Fluorescent gel data were analyzed using the GeneScan software package (Applied Biosystems). For each informative tumor-control pair of reactions (two alleles visible in control samples), an allelic imbalance ratio was calculated: control-allele1:control-allele 2/tumor allele1:tumor allele 2. An allelic imbalance ratio of <0.6 or >1.67 , corresponding to allelic loss in $\geq 50\%$ of tumor cells in an 80% "pure" tumor sample, was considered indicative of LOH.

Di-Deoxy Fingerprinting

Di-deoxy fingerprinting (ddf) was used for initial mutation screening of each of the nine exons of the *PTEN/MMAC1* gene. All exons were PCR amplified separately, using primers that allowed coverage of $>95\%$ of the coding region of *PTEN/MMAC1* (primer sequences available from the authors on request). The PCR contained $1\times$ PCR buffer, 1.5 mM Mg^{2+} , $200\text{ }\mu\text{M}$ of deoxynucleotide triphosphates, $500\text{ }\mu\text{M}$ each of forward and reverse primers, $0.02\text{ U}/\mu\text{L}$ *Taq* polymerase (all Roche Diagnostics), and $2.5\text{ }\mu\text{L}$ of digested tumor sample solution in a total volume of $25\text{ }\mu\text{L}$. PCR conditions were optimized as necessary.

Unincorporated nucleotides and primers were removed from the PCR products by digestion with $0.1\text{ U}/\mu\text{L}$ each of exonuclease I and shrimp alkaline phosphatase (both Amersham Pharmacia Biotech; Uppsala, Sweden; 20). Aliquots of the PCR products were then subjected to a ddf reaction, which is essentially a cycle-sequencing reaction in the presence of a single terminator. These reactions contained $1\times$ PCR buffer, $2\text{ }\mu\text{M}$ of all deoxynucleotide

triphosphates, $20\text{ }\mu\text{M}$ of ddTTP, $1\text{ U}/\mu\text{L}$ *Taq* polymerase (all Roche Diagnostics); $1\text{ }\mu\text{M}$ of forward or reverse primer labeled with 6-FAM, HEX, or TET (Applied Biosystems); and $1.5\text{ }\mu\text{L}$ of cleaned first-round PCR product in a total volume of $10\text{ }\mu\text{L}$.

The ddf reaction products were combined with size standard 500-TAMRA (Applied Biosystems), deionized formamide/loading dye, and 0.1 M NaOH, denatured at 95°C for 5 minutes, quenched on ice, loaded on nondenaturing 0.5% MDE gels (BMA, Rockland, ME), and electrophoresed for 12 hours at 12°C on an automated sequencer (Model 377; Applied Biosystems), using a recirculating refrigerated water bath (Neslab Instruments Inc, Newington, NH,) for cooling. Fluorescent gel data were analyzed with GeneScan software (Applied Biosystems). For each sample, the fragment ladder pattern created by the ddf reaction was examined for mobility shifts compared with control lanes containing reference wild-type DNA, with different peak patterns signifying possible mutations. In addition to several wild-type controls, each ddf gel also included mutant controls derived from cloned *PTEN/MMAC1* exons containing mutations.

PTEN/MMAC1 Sequencing

All 16 chRCC samples and all cRCC and pRCC samples with abnormal ddf patterns were sequenced. For each sequencing reaction, the targets were PCR amplified and cleaned (exonuclease I and shrimp alkaline phosphatase digestion) as described above.

The sequencing reactions contained the following: 3.2 pM of forward or reverse primer, $8\text{ }\mu\text{L}$ of BigDye terminator ready reaction mix (Applied Biosystems), $3\text{--}10\text{ ng}$ of PCR product, and sterile water in a total volume of $20\text{ }\mu\text{L}$. At the end of the cycle sequencing reactions, the extension products were ethanol precipitated, resuspended in gel-loading buffer, denatured at 95°C , and loaded on an automated sequencer (Model 377; Applied Biosystems). After sequencing, the sequences were compared with public database *PTEN/MMAC1* wild-type sequences.

Statistical Analysis and Genotype-Phenotype Comparisons

For each morphotype, the LOH frequencies within the examined regions (centromeric to gene, intragenic, telomeric to gene) were compared with the average LOH rates of the tumors, using contingency tables and χ^2 or Fisher's exact tests for significance testing.

Comparisons of LOH rates and patterns of pRCC and chRCC with each other and with cRCC were also based on contingency tables.

Kaplan-Meier analysis, using the log-rank test for significance testing, was employed to compare the effect of presence or absence of LOH on tumor-specific patient survival.

Stage, grade, and LOH status were also subjected to multivariate analysis, using a Cox model, to estimate their independent effects on tumor-specific patient survival.

RESULTS

Conventional (Clear Cell) Renal Cell Carcinoma

The mean patient age at diagnosis was 59.9 years; 26 patients were female and 54 male. The 5-year mortality rate was 50%, with a median survival interval of 60 months. Twenty-one tumors were classified as TNM Stage 1, 12 were Stage 2, 24 were Stage 3, and 23 were Stage 4. Division of tumors according to Fuhrman grade resulted in 25 Grade 1, 16 Grade 2, 18 Grade 3, and 7 Grade 4 cases. In addition, 14 cases of cRCC showing sarcomatoid dedifferentiation were identified.

10q23.3 LOH was detected in 30 (37.5%) of 80 tumors. Individual markers were informative in a mean of 59% of cases, with a range of 21.2% (*AFMa086wg9*) to 88.7% (*D10S2491*). The average rate of LOH per marker was 16.45%. The most frequent sites of LOH were *D10S541* (37.8%, $P < .001$ compared with the average LOH rate per locus) and *D10S579* (25%, $P < .001$ compared with the average LOH rate per locus; Table 1).

There was no correlation between overall LOH status and tumor stage, grade, or survival. However, LOH at one or both of the intragenic loci correlated strongly with tumor-related death, with 85.7% of such patients dying, whereas only 45.3% of patients without intragenic LOH died ($P = .018$, Fig. 2). The number of cases with intragenic LOH was too small for the multivariate analysis to confirm an independent prognostic role for intragenic LOH.

Abnormal ddf patterns were seen in 16 samples, but sequence analysis of these specimens did not confirm any *PTEN/MMAC1* mutations.

FIGURE 2. Tumor-specific survival of patients without intragenic *PTEN/MMAC1* loss of heterozygosity (LOH; solid line) and of patients with intragenic *PTEN/MMAC1* LOH (broken line).

Papillary Renal Cell Carcinoma

The average patient age at diagnosis was 63.8 years; 8 patients were female and 19 were male. The 5-year mortality rate was 50%, with a median survival interval of 60 months.

Eleven tumors were TNM Stage 1, 8 were Stage 2, 7 were Stage 3, and 1 was Stage 4. Division of tumors according to Fuhrman grade showed 16 Grade 2 and 11 Grade 3 tumors. Sixteen specimens were classified as Type 1, and 11 were classified as Type 2 tumors.

The markers used were informative in a mean of 48.4% of cases. 10q23.3 LOH was detected in 9 (29.6%) of 28 tumors. The average rate of LOH per marker was 28.6%. (Table 1).

There was no correlation between overall or localized LOH and tumor stage, grade, type, or survival.

Although an abnormal ddf pattern were detected in five samples, cycle sequencing of these specimens failed to confirm any *PTEN/MMAC1* mutations.

Table 1. Summary of *PTEN/MMAC1* Locus Loss of Heterozygosity (LOH) Analysis by Marker (in Linkage Order) and Tumor Morphotype

Marker	cRCC ^a			chRCC ^a			pRCC ^a		
	Amplified, N	Informative, N (%)	LOH, N (%)	Amplified, N	Informative, N (%)	LOH, N (%)	Amplified, N	Informative, N (%)	LOH, N (%)
<i>D10S579</i> ^b	30	24 (80)	6 (25)	12	2 (16.6)	1 (25)	21	12 (57.1)	2 (16.6)
<i>D10S215</i> ^b	69	28 (40.5)	4 (14.2)	11	4 (36.3)	2 (50)	21	3 (14.2)	2 (66.6)
<i>D10S2491</i> ^c	80	71 (88.7)	5 (7)	15	15 (100)	8 (57)	23	17 (73.9)	0 (0)
<i>AFMa086wg9</i> ^c	80	17 (21.2)	2 (11.7)	16	2 (12.5)	1 (50)	25	3 (12)	2 (66.6)
<i>D10S608</i> ^d	60	45 (75)	5 (11.1)	13	11 (84.6)	7 (63.6)	22	16 (72.7)	1 (6.25)
<i>D10S541</i> ^d	58	37 (63.7)	14 (37.8)	13	9 (69.2)	4 (44.4)	20	13 (65)	4 (7.6)
<i>AFM280we1</i> ^d	80	35 (43.7)	3 (8.5)	16	7 (43.7)	4 (57.1)	27	12 (44.4)	1 (8.3)

^a Total number of tumors in each group: cRCC = 80; chRCC = 16; pRCC = 27.

^b Located centromeric to *PTEN/MMAC1*.

^c Located within the *PTEN/MMAC1* gene.

^d Located telomeric to *PTEN/MMAC1*.

Chromophobe Renal Cell Carcinoma

The average patient age at diagnosis was 56.4 years; 8 patients were female and 8 were male. The 5-year mortality rate was 56.2%, with a median survival interval of 54 months. One tumor was classified as TNM Stage 1, 7 were Stage 2, 6 were Stage 3, and 2 were Stage 4. Division of tumors according to Fuhrman grade showed 10 to be Grade 2, 5 to be Grade 3, and 1 to be Grade 4.

The seven markers used in this study were informative in a mean of 51.8% of cases, with a range of 12.5% (*AFMa086wg9*) to 100% (*D10S2491*). LOH at 10q23.3 occurred in 87.5% of cases, with the average rate of LOH per marker being 49.6%. The most frequent site of LOH was *D10S608* (63.6%, $P < .001$ compared with average rate per marker; Table 1).

There was no correlation between overall LOH or localized LOH and tumor stage, grade, or survival.

None of 16 cRCC showed evidence of *PTEN/MMAC1* mutations.

Comparisons between cRCC, pRCC, and chRCC

LOH rates around the *PTEN/MMAC1* locus were significantly higher in chRCC than in pRCC or cRCC. With regard to LOH patterns, centromeric markers did not differ statistically among cRCC, chRCC, and pRCC, but intragenic and telomeric markers displayed higher LOH rates for chRCC when compared with the other two morphotypes (Fig. 3). The LOH pattern observed in chRCC showed increasing rates of LOH the further that a marker was located toward the telomer.

DISCUSSION

In this study, we found that deletions at the *PTEN/MMAC1* locus occur in all three major morphotypes renal cell carcinoma. Although chRCC are most commonly affected, cRCC and pRCC also

showed 10q23 LOH in an appreciable minority of cases.

The high rate of LOH at 10q23 in chRCCs detected in this study confirms several previous investigations. The observed LOH pattern, with increased LOH rates toward the telomer, suggests that this is a nonspecific event, probably reflecting the high degree of genomic instability observed in these neoplasms. It is therefore not surprising that LOH at the *PTEN/MMAC1* locus in this morphotype was of no prognostic value. By contrast, the less frequently observed *PTEN/MMAC1* deletions observed in cRCC may be a more specific event, with LOH at one or both of the intragenic markers (*i.e.*, within the actual *PTEN/MMAC1* gene) being significantly associated with tumor-related death. This suggests that in this particular subgroup of cRCCs, inactivation of *PTEN/MMAC1* may be involved in tumor progression. A recently published study of *PTEN/MMAC1* mutations in renal cell carcinoma seems to support this conclusion, with four of five RCC patients (all with cRCC) with tumor *PTEN/MMAC1* mutations dying of their disease (16).

In view of the negative prognostic impact of *PTEN/MMAC1* intragenic LOH in cRCC, the question arises as to why we did not observe any mutations of the *PTEN/MMAC1* gene in our specimens, including those with intragenic LOH. The classical tumor suppressor gene paradigm holds that both alleles of a tumor suppressor gene have to be inactivated before loss of function ensues. This occurs most commonly via a combination of allelic deletion and mutation. There are several possible explanations that such a classical tumor suppressor gene inactivation pattern was not seen in our study. Perhaps our assay simply failed to detect mutations. This seems unlikely, given that ddf generally outperforms other common mutation screening techniques (21). Although we detected several potential mutants by ddf, direct sequencing failed to confirm the ddf screening results in any of these cases. Direct sequencing with BigDye terminators is sensitive enough to reliably detect a polymorphism/mutation if 20–25% of the target DNA contain this polymorphism. In view of this, we should have been able to detect any homozygous or heterozygous mutations in our samples comprising >80% malignant cells. This suggests that if any mutations were present, they were limited to a minor component of the tumor cell population, a possibility that cannot be dismissed as ddf is significantly more sensitive than sequencing and might have therefore correctly detected such samples. Nevertheless, mutations affecting the entire population of cells, or a majority of those cells, were not detected in this study.

Our findings of a lack of correlation between LOH data and the mutation screening results of *PTEN/*

FIGURE 3. Percentage of informative tumors with loss of heterozygosity at markers located centromeric to *PTEN/MMAC1* (left panel), at intragenic *PTEN/MMAC1* markers (center panel), and at markers located telomeric to *PTEN/MMAC1* (right panel).

MMAC1 in renal cell cancer are in agreement with the most recent literature. While Steck *et al.* (11) reported mutations in one of four primary renal carcinomas, later, larger studies have found no, or very few, *PTEN/MMAC1* mutations in RCC (13–17). Furthermore, observations in other human tumor types have also shown few *PTEN/MMAC1* mutations despite high LOH rates at the gene locus (22, 23).

Such data could suggest that another tumor suppressor gene lies within this region or that *PTEN/MMAC1* may be inactivated by mechanisms other than by the combination of mutation and deletion. In prostate adenocarcinoma, as well as in leukemia and lymphoma cell lines, *PTEN/MMAC1* protein expression is frequently down-regulated, or even totally ablated, in the absence of any detectable mutations (24, 25). The precise mechanisms involved in silencing *PTEN/MMAC1* expression in these malignancies are not known, but epigenetic phenomena such as hypermethylation may play a role. It has been suggested that *PTEN/MMAC1* methylation might occur in a subset of prostate carcinomas. However, in other series of prostate tumors, in RCC, and in hematological malignancies, no hypermethylation was observed (14, 24–26).

It is possible that *PTEN/MMAC1* is not a typical tumor suppressor gene and that inactivation of a single allele might be associated with some loss of function. Based on observations in heterozygote *PTEN/MMAC1*+/- knockout mice, it has been suggested that the *PTEN/MMAC1* protein has a dosage effect and that haploinsufficiency of the *PTEN/MMAC1* gene may be responsible for impaired *PTEN/MMAC1* protein expression and activity. In this study, heterozygote *PTEN/MMAC1*+/- knockout mice developed lymph node hyperplasia, splenomegaly, and inflammatory infiltrates in multiple organs, and in the kidney, immune complexes were observed within glomeruli (27). These results are in keeping with findings that heterozygous *PTEN/MMAC1*+/- animals display hyperplastic and dysplastic lesions in many organs in the absence of *PTEN/MMAC1* mutations of the remaining allele (27). Studies in humans with Cowden syndrome, who carry heterozygote *PTEN/MMAC1* germline mutations, have also shown that in a number of cases, the tumors do not have detectable *PTEN/MMAC1* locus LOH (28), indicating that somatic deletion of the wild-type allele has not occurred. Similarly, heterozygous loss of *PTEN/MMAC1* in mouse thyroid and prostate leads to an increase in the proliferative indices of these tissues, again raising the possibility that heterozygous loss alone of *PTEN/MMAC1* may play a role in tumor development and progression (27). Finally, in the most recently published study of *PTEN/MMAC1*

mutations in RCC, at least two, and possibly three (as judged by the published sequencing gels), of the five tumors with *PTEN/MMAC1* mutations appeared to have suffered heterozygote mutations. Nonetheless, only one of these individuals remained alive and free of disease (16).

Our results, showing that *PTEN/MMAC1* seems to be involved in tumor progression in a subgroup of cRCC, suggest that alternative mechanisms of allelic inactivation or a *PTEN/MMAC1* gene dose effect may indeed be occurring in human RCC. These events seem to be limited to the cRCC subtype, although pRCC display a similar *PTEN/MMAC1* LOH pattern, albeit with lower LOH rates. By contrast, chrRCC exhibit nonspecific subtelomeric LOH observed in tumors with high degrees of genomic instability, and it is unlikely that *PTEN/MMAC1* inactivation plays a role in pathogenesis or progression of this morphotype.

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