

# Analysis of PTEN gene by fluorescent *in situ* hybridization in renal cell carcinoma

## *Análise do gene PTEN por hibridização in situ fluorescente no carcinoma de células renais*

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### A B S T R A C T

**Objective:** To evaluate the frequency of deletion of the PTEN gene in renal cell carcinoma (RCC) and its impact on the rates of overall and disease-free survival. **Methods:** We analyzed 110 patients with renal cell carcinoma who underwent radical or partial nephrectomy between 1980 and 2007. In 53 cases it was possible to analyse the PTEN gene by the method of fluorescent *in situ* hybridization using the technique of tissue microarray. For statistical analysis, patients were classified in two groups according to the presence or absence of the deletion. **Results:** The mean follow-up time was 41.9 months. Hemizygous deletion was detected in 18 patients (33.9%), while the homozygous one was present in three (5.6%). Deletion was present in approximately 40% of the analyzed cases. Monosomy and trisomy were detected in nine (17%) and two patients (3.8%), respectively. In 21 patients (39.6%) the analysis of the PTEN gene by *in situ* hybridization was normal. There were no statistically significant differences in overall ( $p = 0.468$ ) and disease-free ( $p = 0.344$ ) survival rates between patients with or without deletion. Factors which were independent for overall survival: TNM clinical stage, symptoms at diagnosis, high Fuhrmann grade, performance status (ECOG) and tumor recurrence. Disease-free survival was influenced only by the clinical TNM stage. **Conclusion:** Deletion of the PTEN gene in RCC was detected with a frequency of approximately 40% and its presence was not determinant of lower survival rates, the traditional prognostic factors remaining as determinants of outcome.

**Key words:** Gene deletion. PTEN phosphohydrolase. In situ hybridization, fluorescence. Carcinoma, renal cell. Survival rate.

### INTRODUCTION

The renal cell carcinoma (RCC) is among the ten most frequent malignancies in the U.S.<sup>1</sup>. Despite the increase in diagnosis of smaller, asymptomatic renal tumors, nearly 30% of patients have metastases at diagnosis and other 30% will develop metastases during the course of the disease, even when it is localized<sup>1-4</sup>.

Biomolecular factors have been studied to aid in clinical and pathological staging, identifying different genes and proteins capable of predicting the risk of progression or death by the disease and identify patients who may have a better response to treatment<sup>3</sup>.

Among the genes studied, PTEN (phosphatase with tensin homology deleted in chromosome 10) is a tumor suppressor gene located on chromosome 10q23 and may be inactivated due to mutations and deletions, these being found in various solid malignancies<sup>2</sup>. In RCC, a deletion or mutation of PTEN, as well as its

immunohistochemistry low expression, are associated with the invasive and metastatic phenotype of tumor<sup>2,4-8</sup>.

The aim of this study was to analyze the PTEN gene via tissue microarray (TMA) by the technique of fluorescence *in situ* hybridization (FISH), determining the frequency of deletion and its impact on the rates of overall and disease free survival.

### METHODS

We retrospectively analyzed 53 patients with renal cell carcinoma, metastatic or not at diagnosis, and subjected to surgical treatment between 1980 and 2007. The research of fluorescent *in situ* hybridization (FISH) of the PTEN gene was initially performed in specimens from 110 patients, which had been submitted to TMA, but only in 53 samples (48.2%) it was possible to perform the proposed reaction. Different factors, such as the presence

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of artifacts in the slide obtained from TMA or cell rareness, were decisive for the proposed reaction not to occur.

The classification of Fuhrman *et al.*<sup>9</sup> was used, and grades I and II tumors were considered low-grade tumors, grades III and IV being considered as high grade. To define the histological subtype, the classification of the World Health Organization<sup>10</sup> was used. Patients were analyzed in two groups: stage I and II (localized disease) versus III and IV (locally advanced or metastatic disease) according to the 2004 TNM classification<sup>11</sup>. Survival rates were calculated by the Kaplan-Meier technique and comparisons between them were carried out with Log Rank test. For the multivariate analysis of survival the model employed the Cox proportional hazards. For the analysis of Disease-free survival, patients with metastases at diagnosis or positive surgical margins were excluded from the study.

### Tissue microarray (TMA)

The 53 tumors designated by their corresponding registration pathology report numbers, previously selected and classified, were included in the construction of a TMA receptor paraffin block (Beecher Instruments, Silver Spring, MD) from samples of the original donor block, with 1mm diameter needle (TMArrayer punch MP10-1.0mm) after the previous choice and marking of the neoplasm representative area from the original hematoxylin and eosin slide.

We performed punctures in duplicate (two fragments each case) of the donor and receptor paraffin blocks, thus containing two different areas of the tumor in each case. Histological sections with 3 to 4  $\mu$ m thickness of the receptor paraffin block were also performed until its end, yielding a total of 110 sequentially numbered slides, starting from one. To better represent tumor heterogeneity we used more than one slide with samples in duplicate.

### Fluorescent in situ hybridization

The detection of structural changes or alterations in the number of chromosomes occupying the locus was accomplished by reactions of FISH, using a probe of 368kb with specific affinity for the 10q23 region of chromosome 10, containing sequences that identify the 5' and 3' portions of the PTEN gene. The PTEN gene was considered normal by the presence of two red signals (10q23/PTEN locus) and two green signals (CEP 10).

The presence of hemizygous deletion was defined when one allele was partially or completely modified, the wild-type gene being present, ie when there was more than 24% of the tumor containing only one PTEN gene locus and the presence of the control signal. Homozygous deletion was defined by reading the slides, when the PTEN gene loci were absent and the control group signal was present in more than 30% of the cells.

Regarding number, trisomy was defined when, besides the presence of two chromosomes and their alleles, there was an extra chromosome, ie, when during reading there was more than 15% of the tumor containing three

PTEN loci and the presence of three signals in control. Monosomy was defined when only one chromosome was present, occupying the locus 10, and the reading showed over 23% of tumor containing a signal on the PTEN locus and the presence of the control signal.

For statistical analysis of overall and disease-free survival, two groups of patients were defined according to the results of the reaction of FISH. Patients who displayed hemizygous deletion, homozygous deletion or monosomy at hybridization were assigned to the deletion group. Reaction of in situ hybridization with findings of trisomy or normality comprised the no-deletion group.

## RESULTS

The mean age was 54.9 years, male patients predominating (61.8%). At the end of the study, there were 68 patients (61.8%) alive and without disease, seven (6.4%) alive with disease, 35 deaths (31.7%) and four patients (3.6%) lost to follow-up. Table 1 shows the main epidemiological, clinical and pathological features of the 110 patients analyzed.

Among the specimens positive for deletion, 18 (33.9%) showed hemizygous deletion and 3 (5.6%) homozygous. In 21 (39.6%), PTEN deletion was not found. Monosomy and trisomy of the gene were detected in 11 specimens (20.7%), nine (17%) and two (3.8%), respectively. Among the 53 slides with positive FISH, 30 (56.6%) had deletion and 23 (43.4%) did not. Figure 1 demonstrates the findings to the FISH reaction.

The overall survival rates at five years were not influenced by the presence or absence of deletion, and were respectively 75.7% and 82.8% ( $p = 0.468$ ). As well as overall survival, there were no statistically significant differences in the presence or absence of deletion in relation to disease-free survival, respectively 70.3% and 84.7% ( $p = 0.344$ ), although we have observed a tendency for higher overall and disease-free survival of patients without the deletion at the FISH reaction. Figure 2 shows the survival rate according to the in situ hybridization of PTEN.

Multivariate analysis showed that overall survival was independently influenced by the following variables: clinical stage IV (HR = 7.095,  $p = 0.011$ ), ECOG 1 and 2 (HR = 0.28,  $p = 0.01$ ), symptoms at diagnosis (HR = 3.380,  $p = 0.037$ ), high Fuhrman grade (HR 2.421,  $p = 0.057$ ) and tumor recurrence (HR = 3.380,  $p = 0.029$ ). Disease-free survival was only influenced by clinical stages II and III (HR = 4.34 and 12.85,  $p = 0.02$  and 0.01, respectively).

## DISCUSSION

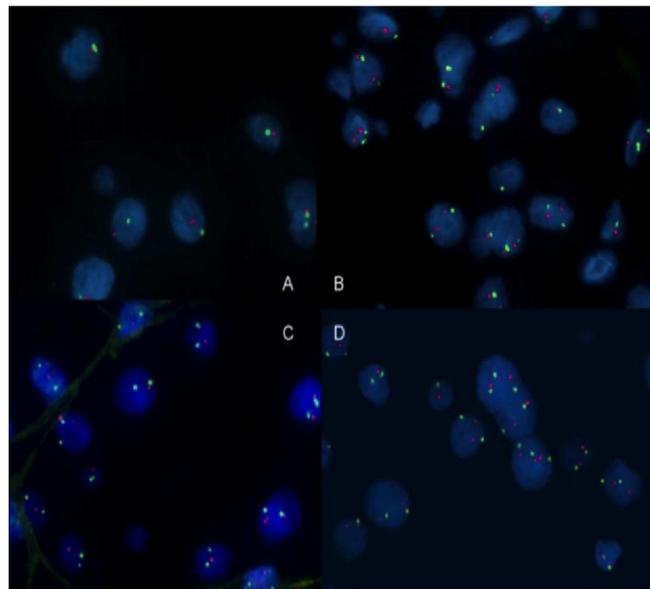
Despite the routine use of clinical and pathological criteria in assessing the prognosis of patients with RCC, many develop metastases during follow-up,

**Tabela 1** - Variáveis epidemiológicas, clínicas e anatomopatológicas dos 53 pacientes analisados.

Variável	Categoria	N	%
ECOG	0	84	76,4
	1 – 2	26	23,6
Metástase ao diagnóstico	Não	94	85,5
	Sim	16	14,5
Sintomatologia ao diagnóstico	Não	66	60
	Sim	44	40
Grau Fuhrman	Baixo grau	65	59,1
	Alto grau	45	40,9
EC TNM 2004	I e II	68	61,8
	III e IV	42	38,2
Recorrência tumoral	Não	79	71,8
	Sim	31	28,2

demonstrating the need to identify new factors that can better predict the evolution of these patients <sup>1,3</sup>.

The PTEN/MMCA1 gene is located on chromosome 10q23, regulating cell migration, proliferation and apoptosis, acting by a mechanism of down regulation of the PI3K (phosphatidylinositol 3 OH kinase) and of the Akt protein. In RCC, low immunohistochemical expression of PTEN has been associated with high grade tumors and lower survival rates <sup>1,7</sup>.

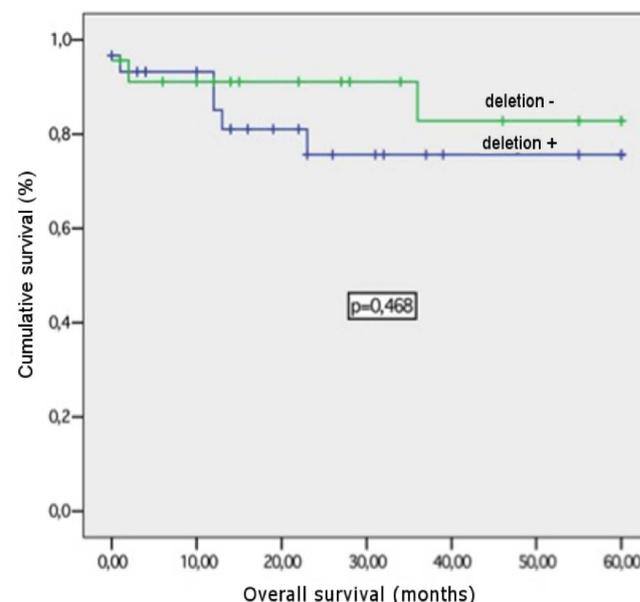


**Figure 1** - A) Monosomy of chromosome 10 identified by the presence of a red signal (10q23/PTEN locus) and a green light (CEP 10); B) Normal representation of the PTEN gene by the presence of two red signals (10q23/PTEN locus) and two green signals (CEP 10); C) representation of hemizygous deletion by the presence of a red signal (10q23/PTEN locus) and two green signals (10q23/PTEN locus); and D) trisomy of chromosome 10, represented by the presence of three red signals (10q23/PTEN locus) and three green signals (CEP 10).

The technique of fluorescent *in situ* hybridization allows the evaluation of genes by the addition of specific probes labeled with fluorescent material, identifying numerical changes (chromosomal gains or losses, amplifications, deletions) or structural abnormalities (translocation), being superior to other techniques for suffering no influence by tumor heterogeneity.

Cell rareness, concentration of the probe, post-hybridization washing and origin of the blocked paraffin material <sup>12</sup> are limitations of the technique and may interfere with the analysis, as they did in the present study, causing the exclusion of 57 cases from an initial series of 110.

Few studies have analyzed the status of the PTEN gene when there are genomic changes detected by *in situ*



**Figure 2** - Survival rates according to in situ hybridization of PTEN.

hybridization. The methodology employed by the studies differ, and the samples sizes are small. In this study, we used the same methodology proposed by Korshunov *et al.*<sup>13</sup> and Ventura *et al.*<sup>14</sup>, using higher cut-off values for homozygous deletion, to precisely show those cases where the gene was actually absent at FISH.

Monosomy of PTEN was found with a frequency of 17%, higher than that described in the literature, close to 5%<sup>15</sup>. For Speicher *et al.*<sup>16</sup>, monosomy in PTEN is a rare event in the clear cell subtype, but may be detected in up to 40% of cases in the chromophobe subtype. Thus, the inclusion of subtypes different from the clear cell RCCs in the study may explain the higher frequency found. In the methodology, the use of a higher cut-off value and 23% for the detection of monosomy allowed the detection of cases that actually had monosomy, thus preventing false-positive cases.

Considering RCC, Dal *et al.* Clin.<sup>17</sup> evaluated the frequency of PTEN trisomy through *in situ* hybridization in 17 specimens of patients undergoing nephrectomy. Trisomy was found in five patients (30%). In the 53 analyzed by hybridization in this study, trisomy was infrequent, present in only two patients (3.8%).

In 41 specimens analyzed by *in situ* hybridization, Presti *et al.*<sup>18</sup> demonstrated that

chromosomal abnormalities are more frequent in RCC on chromosome 3 and corresponded to 29% of the cases studied. Chromosomes 8, 10 and 14q were altered in 20% of cases. The impact of the deletion in survival rates was not assessed; however, the authors demonstrated that deletion of the genes was not associated with Fuhrmann high-grade tumors.

In Glioblastoma Multiforme, deletion of PTEN was detected with a frequency of 61% and was determinant of lower overall survival in 44 cases<sup>13</sup>. In this study, we identified approximately 40% deletion, noting lower rates of overall survival and disease-free survival for patients with molecular alteration at FISH. Considering the major technical difficulties inherent to the reaction of *in situ* hybridization, bigger sample sizes are needed to be able to demonstrate with greater significance the observed differences and thus the negative impact of the presence of deletion of PTEN in the evolution of patients with RCC.

In conclusion, deletion of the PTEN gene in RCC was detected with a frequency of approximately 40% and its presence was not determinant of lower survival rates, the traditional prognostic factors remaining as determinants of outcome.

## R E S U M O

**Objetivo:** avaliar a frequência de deleção do gene PTEN no carcinoma de células renais e o impacto da deleção nas taxas de sobrevida global e livre de doença. **Métodos:** foram analisados 110 pacientes portadores de carcinoma de células renais submetidos à nefrectomia radical ou parcial entre os anos de 1980 e 2007. Em 53 casos foi possível a análise do gene PTEN pelo método de hibridização *in situ* fluorescente através da técnica de "tissue microarray". Para a análise estatística, os pacientes foram classificados em dois grupos, de acordo com a presença ou ausência de deleção. **Resultados:** o tempo médio de seguimento foi de 41,9 meses. Deleção hemizigótica foi identificada em 18 pacientes (33,9%), ao passo que deleção homozigótica esteve presente em três (5,6%). Em aproximadamente 40% dos casos analisados havia deleção. Monossomia e trissomia foram detectadas, respectivamente, em nove (17%) e dois pacientes (3,8%). Em 21 pacientes (39,6%), a análise por hibridização *in situ* do gene PTEN foi normal. Não houve diferenças estatisticamente significativas nas taxas de sobrevida global ( $p=0,468$ ) e livre de doença ( $p=0,344$ ) entre os pacientes portadores ou não de deleção. Foram fatores independentes para a sobrevida global: estágio clínico TNM, sintomatologia ao diagnóstico, alto grau de Fuhrmann performance status (Ecog) e recorrência tumoral. A livre de doença foi influenciada unicamente pelo estágio clínico TNM. **Conclusão:** deleção do gene PTEN no CCR foi detectada com frequência de aproximadamente 40% e sua presença não foi determinante de menores taxas de sobrevida, permanecendo os fatores prognósticos tradicionais como determinantes da evolução dos pacientes.

**Descritores:** Deleção de genes. PTEN Fosfo-hidrolase. Hibridização *in situ* fluorescente. Carcinoma de células renais. Taxa de sobrevida.

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