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Monosomy of Chromosome 9 is Associated with Higher Grade, Advanced Stage and Adverse Outcome in Clear Cell Renal Cell Carcinoma

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Abstract

The objective of the study was to evaluate the frequency and the outcomes of whole chromosome 9 loss in 103 patients with clear cell renal cell carcinoma (ccRCC) using Single nucleotide polymorphism-based chromosome microarray (CMA) analysis. Our study, demonstrated chromosome 9 loss is associated with higher grade, advanced stage and poor outcome in ccRCC and can potentially be used as a major prognostic predictor in ccRCC patients.

Background: Clear cell renal cell carcinoma (ccRCC) is one of the most common malignancies in humans and is usually associated with poor outcomes. Cancers are considered to be genetic diseases. Therefore, a better understanding of genetic alterations that are related to disease progression or poor prognosis can help to more precisely identify high-risk patients and treat them more effectively. The aim of this study was to examine the frequency of whole chromosome 9 loss (monosomy of chromosome 9) and its prognostic value in patients with ccRCC.

METHODS: Single nucleotide polymorphism-based CMA analysis was performed on 103 resected specimens from ccRCC patients who had undergone partial or radical nephrectomy between January 2002 and March 2017 at Fox Chase Cancer Center (FCCC). Monosomy 9 was correlated with clinicopathological parameters and recurrence-free survival.

RESULTS: Chromosome 9 loss was detected in 31 (30%) out of 103 tumors. Tumors with chromosome 9 loss had higher histologic grade (3 and 4, $p<0.001$) and pathological stage ($P<0.001$). In 59 non-metastatic ccRCC patients, chromosome 9 loss was also associated with higher recurrence rate and shorter recurrence-free survival (RFS) [(12-month RFS=77.8% (95%CI:36.5–93.9%) for chromosome 9 loss vs 95.7%, (95%CI:84.0–98.9%) for no loss, $p=0.002$].

CONCLUSIONS: Chromosome 9 loss was found in 30% of ccRCC patients and correlated with higher grade, advanced stage and shorter RFS in patients with Stage I-III ccRCC.

Keywords

Clear cell renal cell carcinoma; kidney cancer; chromosome 9; outcome; recurrence

1. Introduction

Renal cell carcinoma (RCC) is the second most frequent urologic malignancy after bladder and is one of the most aggressive urological malignancies, with a median survival rate of only 13 months.^{1, 2} The metastasis rate in RCC is 20–30% at the time of diagnosis, and 20–50% of patients with localized disease may show metastasis after nephrectomy.³

The most important prognostic factors after surgery are TNM (Tumor-Node-Metastasis) staging and Fuhrman grade.^{4,5,6} However, by increasing the number of diagnosed small renal tumors in recent years, it has been shown that the traditional methods for predicting prognosis such as pathological and imaging parameters cannot accurately predict the natural history of the emerging small tumors. Therefore, there is an urgent need to find additional alternative methods for characterization and prediction of behavior of these tumors.⁷

Cancers are often considered a disease of the genome marked by clonal genetic alterations.⁸ The patterns of cytogenetic alterations differ in malignancies depending on the type of tumor and its stage of development. Typically, early stage tumors exhibit fewer cytogenetic alterations than tumors that are more advanced.⁹ Inactivating germline mutations of the *VHL* tumor suppressor gene, on chromosome arm 3p, are a frequent early molecular change that predispose patients to develop clear cell RCC (ccRCC), especially in the form of numerous bilateral tumors.^{10,11,12}

Clinicopathological correlations and prognostic implications of copy number aberrations (CNAs) in ccRCC have been reported in some studies.^{13,14} Deletion of chromosome 9 is one of the most common genomic imbalances detected in ccRCC.^{10,15} Most previous studies have described an association between 9p deletions and its prognosis.^{16,18,19,20,21} However, the importance and frequency of the whole chromosome 9 loss in ccRCC is less well appreciated. Furthermore, almost all such studies reported to date have used the older histologic grading system (Fuhrman Nuclear Grade), whereas we have adapted the current World Health Organization (WHO)/International Society of Urological Pathology (ISUP) histologic grading system in the current study. In addition, most of the previous reports have utilized conventional cytogenetic, microsatellite and FISH methods. One published study has used comparative genomic hybridization (CGH) analysis to investigate the significance

of chromosome 9 in ccRCC,¹⁷ but this study utilized the Fuhrman grading system. In addition, because of the way RCC cases are selected for chromosome microarray (CMA) analysis in our institution, this study is enriched in high grade tumors, giving us a better opportunity to uncover the genomic imbalances in high grade ccRCC.

The purpose of the present study was to assess the frequency of whole chromosome 9 loss by CMA analysis in patients with ccRCC who underwent partial or radical nephrectomy and to correlate this finding with patient survival and clinicopathological features. Our results suggest that chromosome 9 loss is almost entirely restricted to higher grade tumors and is associated with advanced stage and poor outcome in ccRCC. The important candidate tumor suppressor genes located on chromosome 9 are also discussed.

2. Materials and Methods

2.1. Patient selection

The Institutional Review Board of FCCC approved this study. The prospectively-maintained FCCC kidney cancer database was used to identify 103 ccRCC patients with partial or radical nephrectomy who had undergone SNP-based CMA analysis between January 2002 and March 2017. All tumor specimens were re-reviewed by an experienced urologic pathologist (TAS). The grading system proposed by the ISUP (Vancouver) and later adopted by the WHO in 2016¹⁸ was used for histological grading the tumors. The patients' clinicopathological data, including patient age at the time of surgery, gender, date of surgery, clinical and pathological stages, and follow-up data were extracted from the prospectively maintained kidney cancer database.

2.2. Chromosome Microarray Analysis

Pathological review of hematoxylin and eosin-stained tissue directly mirroring the area used for CMA analysis was performed to ensure that the region of the tumor used was as phenotypically homogeneous as possible and to maximize the amount of tumor present in the tissue sample while minimizing the presence of stroma, normal tissue, and necrosis. Individual ccRCC samples contained variable amounts of tumor cells, usually representing at least 60% of all cells in the area used for CMA. Among cases with monosomy 9, loss of 9 was usually observed in a mosaic state representing 40–90% of the cells. Total genomic DNA was extracted with a Qiagen Gentra DNA kit, and CMA analysis was performed with according to the manufacturer's protocols. Samples obtained prior to November of 2010 (n=17) were analyzed using Affymetrix Genome-wide Human SNP array 6.0. Samples obtained between November 2010 and March 2012 were analyzed using Affymetrix Cytogenetics 2.7M arrays, and samples examined after April 2012 were analyzed using Affymetrix CytoScan HD arrays. All samples were processed for clinical purposes; therefore, extensive validation was performed to ensure uniformity between all analytic platforms to conform to CLIA (Clinical Laboratory Improvement Amendments) standards. The microarrays were scanned with an Affymetrix GeneChip scanner (3000 7G), the signal intensities were analyzed with Affymetrix GeneChip Command Console Software, and copy number and LOH analysis were performed with Genotyping Console (GTC) or Chromosome Analysis Suite (ChAS) with default settings.

2.3. Statistical Analysis

Chromosome 9 loss for each sample was determined by a pathologist (RN) by reviewing the detailed CMA summary results. Three tumors with partial chromosome 9 alteration were excluded from the comparison of total loss vs no loss. Differences in chromosome 9 loss status by demographic and tumor characteristics were assessed using Chi-square tests or Fisher's exact tests; age distributions were described with median and interquartile range (IQR) and compared with Wilcoxon rank sum tests. Recurrence was defined as any recurrence (local or distant) following complete resection of localized disease. For recurrence free survival analyses in the non-metastatic patients, the outcome was calculated from the time of surgery to the time of recurrence or death from disease, with patients alive without recurrence or who died of other causes being censored at the date of their last follow-up or death. In this cohort, all patients who died from kidney cancer-related causes had local or distant recurrence prior to death. Patients treated as of December 31, 2016 were included in the recurrence analytic cohort. Recurrence-free survival was estimated using Kaplan Meier methods for the chromosome 9 loss groups and compared with log rank tests. Cox proportional hazards regression was used to estimate the HR on time to recurrence or death from ccRCC and chromosome 9 loss, adjusting for risk factors include grade, stage, and age as continuous variables. Due to the low number of events, risk factors were assessed separately. SAS statistical software version 9.4 (Cary, NC) was used for analyses, with p-values of < 0.05 considered significant.

3. Results

Of 103 tumors, 69 (67%) had no chromosome 9 loss, three tumors (3%) showed partial chromosome 9 alterations (2 tumors with 9p deletions and 1 tumor with 9q deletion), and 31 (30%) showed monosomy of chromosome 9 including one tumor with copy neutral loss of heterozygosity (cnLOH) of whole chromosome 9 (Figure 1). The median age at surgery for the 103 patients was 59 years (range 29–84) and included 70 men (68%), which is typical of RCC. Associations between patients' clinicopathological features and the status of chromosome 9 are shown in Table 1. Chromosome 9 loss was observed in only one of 32 tumors (3%) with low histologic grade (grade 1–2). In contrast, chromosome 9 loss was seen in 30/68 (44%) of high grade tumors (Grade 3: 11 of 34 (32%), grade 4: 19 of 34 (56%), $p < 0.001$). Chromosome 9 loss was also significantly associated with pathological stage (range for proportion with total loss: pStage I = 8% to pStage IV=54%, $p < 0.001$). Median age at surgery in the total loss group was 61 years (IQR:54–67) compared to 58 years (IQR: 51–66) in patients without chromosome 9 loss ($p=0.23$).

Survival outcomes were evaluated for 99 patients who had surgery prior to 2017. There were 24 deaths during the follow-up period, including 18 cause-specific deaths. Of those alive, the median follow-up from surgery was 37.9 months (IQR: 17.7–64.6 months). In 59 pStage I–III patients, there were 11 with recurrence, (5 of 9 patients with total chromosome 9 loss; 6 of 50 patients with no loss); all of the cause-specific deaths had recurrence. Chromosome 9 loss was associated with lower recurrence free survival :12-month RFS was 77.8% (95%CI:36.5–93.9%) for chromosome 9 total loss compared to 95.7%, (95%CI:84.0–98.9%) for no loss, $p=0.002$ (Figure 2), with a hazard ratio (HR) estimate of 6.1 (95% CI: 1.7–21.9).

In additional separate multivariable models, the association of monosomy of chromosome 9 with recurrence was independent of pStage and age (adjusted HR=6.3 (95%CI 1.6–24.3) and 6.2 (95%CI 1.7–22.2), respectively).

4. Discussion:

In this study, we found that chromosome 9 loss is associated with higher-grade, advanced-stage tumors and shorter DFS in patients with non-metastatic ccRCC.

We were surprised to find loss of whole chromosome 9 in 30% of cases. Several groups have reported frequent deletion of 9p in ccRCC pathogenesis,^{15,10,19,20} with the incidence of this finding ranging between 14%²¹ and 44%,⁷ but the actual frequency of whole chromosome 9 loss in each grade has not been well established. In our study, only a small proportion of ccRCCs with alterations of chromosome 9 (i.e., 2 of 34) were deletions of 9p; the remainder involved whole chromosome copy number losses or, in one case, cnLOH of the entire chromosome 9.

The variability of the reported frequency of chromosome 9p deletion is due, in part, to the different technologies that were used in these studies. FISH and karyotyping were the most common methods that have been used in previous studies, and these techniques have limited resolution. Furthermore, intratumor heterogeneity is a very well-known phenomenon in cancers and sampling bias such as using small biopsy cores or tissue microarrays could also contribute in this variability. Additionally, our cohort is enriched with high-risk pathology, with 69% of tumors characterized as having high nuclear grades (3 or 4), and 34% of the patients presenting with advanced disease (Stage 4).

One of the main findings of our study was the correlation between chromosome 9 loss and higher recurrence rate in ccRCC. The prognostic significance of chromosome 9p loss has been shown in several studies.^{7,15, 22,23,24,28,29,30} However, we believe loss of entire chromosome 9 is associated to progression due to not only loss of the genes located on chromosome 9p, but also to loss of genes located on chromosome 9q which have a crucial role in renal cell metabolism and proliferation pathways.²⁷

Several candidate tumor suppressor genes on chromosome 9p have been proposed to play a role in the progression of ccRCC. The cyclin-dependent kinase inhibitor 2A locus (*CDKN2A*) at chromosome 9p21.3 encodes both the p16(INK4A) and p14(ARF) proteins, which regulate the critical Rb and p53 cell cycle regulatory pathways, respectively.⁷ During the cell cycle, these proteins regulate cell cycle progression, for example p16(INK4A) plays an essential role in inducing G1 cell cycle arrest by inhibiting the phosphorylation of the Rb protein by the cyclin-dependent kinases CDK4 and CDK6.²⁵ Other tumor suppressor genes such *CDKN2B* and *MTAP* are located near *CDKN2A* at 9p21.3. *CDKN2B* encodes p15(INK4B), another tumor suppressor. p15(INK4B) is a negative regulator of CDK6 and CDK4. Somatic inactivation of *CDKN2B* through allelic loss or promoter hypermethylation has been found in many human neoplasms, including leukemia, lymphoma, and myelodysplastic syndrome.²⁶ The *MTAP* gene encodes methylthioadenosine phosphorylase, a critical enzyme in the methionine salvage pathway. Loss of the *MTAP* gene is observed at

a high frequency in numerous tumor types, and MTAP has tumor suppressor functions independent of CDKN2A and CDKN2B loss.²⁷

Regarding the long arm of chromosome 9 (9q), the *FBP1* locus resides at chromosome band 9q22, and its loss is associated with poor prognosis in patients with ccRCC.²⁸ *FBP1* encodes the gluconeogenic enzyme fructose-1,6-bisphosphatase 1, and Li et al. showed that FBP1 normally plays a key role in inhibiting ccRCC progression and tumorigenesis.²⁴ However, they showed that lower FBP1 expression correlated significantly with advanced tumor stage and worse patient prognosis in ccRCC. Their work indicated that FBP1 inhibits ccRCC progression by two mechanisms: 1) inhibiting a potential “Warburg effect” by counteracting glycolytic flux in renal tubular epithelial cells, the possible ccRCC cell of origin, and 2) inhibiting nuclear hypoxia inducible factor (HIF) function through a direct interaction in pVHL-deficient ccRCC cells.²⁹

Another candidate 9q gene that is important in cell-cell interactions is *NOTCH1*, which is located at chromosome 9q34.3. Notch refers to a member of family of cell-surface receptors that interfere in various cellular processes including differentiation, proliferation, and apoptosis.³⁰ Based on the tumor type, Notch family members may act as oncogenically or as tumor suppressors.³¹ The tumor suppressor gene *TSC1*, located at chromosome 9q34.13, is another noteworthy candidate gene. LOH of *TSC1* in RCC identified in more than 30% of specimens reviewed in The Cancer Genome Atlas (TCGA) project.³² In a study with mouse models, deletion of *Tsc1/Tsc2* which are located upstream of mTORC1, leads to the progression of a variety of tumors, including lung, kidney and prostate carcinomas as well as mesotheliomas.^{33, 34}

Additionally, monosomy 9 results in loss of one copy of multiple tumor suppressor genes, e.g., *CDKN2A/B*, *TSC1*, *FBP1*, *PTCH1* and *NOTCH1*, distributed throughout the short and long arms of this chromosome. Their collective losses may thereby promote a strong phenotype as a form of collaborative haploinsufficiency, perhaps similar to the cooperativity that might exist between *VHL*, *SETD2*, *BAP1* and *PBRM1* distributed over chromosome arm 3p in ccRCC.

Our study has several potential limitations, including the retrospective nature of the study and relatively small sample size. Despite these shortcomings, ours is one of the only studies that have used both chromosome microarray analysis method and the current WHO/ISUP histologic grading system to investigate the significance of chromosome 9 loss in ccRCC.

In conclusion, our findings suggest that whole chromosome 9 loss is correlated with higher grade, advanced stage and inferior disease outcome in ccRCC and could be used as a major prognostic predictor in ccRCC patients. Our finding emphasizes the importance of CMA analysis in the workup of ccRCC patients and could be beneficial in predicting the tumor behavior and choosing suitable candidates for more aggressive treatment strategies. Future studies are warranted to explore the role of the various tumor suppressor genes on chromosome 9 in ccRCC progression.

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Clinical Practice Points

- Most of the published studies have described an association between 9p deletions and its prognosis. However, the importance and frequency of the whole chromosome 9 loss in ccRCC is not well appreciated.
- In this study by using single nucleotide polymorphism-based chromosome microarray analysis, the majority of cases (30%) showed whole chromosome 9 loss which correlated with higher grade, advanced stage and shorter RFS in patients with Stage I-III ccRCC.
- This finding suggests that the association of entire chromosome 9 loss to progression might be not only due to loss of the genes located on chromosome 9p, but also to loss of genes located on chromosome 9q which have a crucial role in renal cell metabolism and proliferation pathways.
- Taken together, this study emphasizes the importance of CMA analysis in the workup of ccRCC patients and suggests that whole chromosome 9 loss can be used as a major prognostic predictor in ccRCC patients.

Figure 1. Chromosome microarray analysis (CMA) analysis of two representative ccRCCs with alterations of chromosome 9.

Note that for each tumor, the upper panel shows the copy number analysis, whereas the lower panel shows B-allele frequency (BAF) analysis. Normally, there are three “bands” in the allele analysis panel; whereas four bands indicates mosaic LOH. **A**, Tumor showing copy neutral LOH (cnLOH) of whole chromosome 3 and whole chromosome 9 in a mosaic state representing about 40% of the cells. **B**, Another ccRCC showing losses of short arm p of chromosome 3 and whole chromosome 9 in a mosaic state representing about 50–60% of the cells.

Figure 2.
Kaplan-Meier estimates of recurrence-free survival from date of surgery by Chromosome 9
Total Loss status Stage I-III ccRCC patients.

Table 1:

Clinicopathological features and the status of chromosome 9

	All, N=100 patients [*]				pStage I-III, n=59 patients			
	<u>C9 Total Loss</u>				<u>C9 Total Loss</u>			
	N	n	%	P ^{**}	N	n	%	P ^{**}
<i>All</i>	100	31	31.0		59	9	15.3	
<i>Age at Surgery (yrs)</i>				0.70				0.49
<i>28-49</i>	19	4	21.1		16	1	6.3	
<i>50-59</i>	34	10	29.4		17	3	17.6	
<i>60-69</i>	34	12	35.3		16	2	12.5	
<i>70-84</i>	13	5	38.5		10	3	30.0	
<i>Gender</i>				0.57				0.71
<i>Female</i>	33	9	27.3		17	3	17.6	
<i>Male</i>	67	22	32.8		42	6	14.3	
<i>Race</i>				0.64				0.40
<i>White</i>	95	29	30.5		56	8	14.3	
<i>Other or unknown</i>	5	2	40.0		3	1	33.3	
<i>Grade</i>				<0.001				0.003
<i>1</i>	4	0	0		4	0	0	
<i>2</i>	28	1	3.6		24	0	0	
<i>3</i>	34	11	32.4		20	4	20.0	
<i>4</i>	34	19	55.9		11	5	45.5	
<i>pStage</i>				<0.001				0.18
<i>I</i>	36	3	8.3		36	3	8.3	
<i>II</i>	7	2	28.6		7	2	28.6	
<i>III</i>	18	5	27.8		16	4	25.0	
<i>IV</i>	39	21	53.8					

* excludes 3 patients with partial Chromosome 9 alteration

** p-value from Chi-square test or Fisher's exact test

Abbreviations: c9=Chromosome 9, yrs =years, pStage=pathological Stage