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APPLICATION OF TISSUE MICROARRAY TECHNIQUE IN THE EVALUATION OF CHROMOSOMAL ABNORMALITIES OF CLEAR CELL RENAL CELL CARCINOMA

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INTRODUCTION

Renal cell carcinoma (RCC) accounts for approximately 3% of all adult malignancies and is the most lethal genitourinary tumors.

More than 40% of RCC patients die of the disease, and the vast majority of them are represented by clear-cell renal cell carcinoma (ccRCC), whereas the mortality rates for other urologic cancers, such as prostate cancer and bladder cancer, are approximately 20%. (1)

Although there are many types of emerging treatments, including radiofrequency ablation, interventional therapy, immunological therapy, and targeted therapy, surgery is still the most effective treatment for this disease. (2)

In contrast, immunotherapy is actually the only effective therapeutic strategy for patients with metastatic ccRCC, but, unfortunately, it is characterized by high levels of toxicity and low rates of response. (3)

As the main histotypes of RCC seem to be resistant to current forms of adjuvant therapy, attempts should be made to better understand the biological basis of tumor development and progression, to identify specific target therapies, as well as to better define markers for assessing prognosis or predicting therapy outcome.

Previous studies have shown that RCC histological subtypes are genetically and biologically different. These include clear cell (80%), papillary (about 10%), chromophobe (5%), and collecting duct (1%) carcinomas, with patients with ccRCC having a significantly poorer survival than patients with papillary or chromophobe variants. (4) The role that specific genetic alterations play in determining prognosis, phenotype-genotype correlation, and clinical patterns needs to be investigated for each of these tumor types.

Initiation and progression of cancer is due to genetic alterations. Recent studies characterizing genetic aberrations in RCC implicated a number of chromosomal loci as important in cancer development. It has been suggested that an accumulation of genetic events is responsible for tumor progression in RCC, although the details of genetic changes and their order of occurrence in renal tumorigenesis are not well understood. (5,6)

Genes potentially involved in kidney cancer include the von Hippel-Lindau gene on chromosome 3p, the epidermal growth factor receptor gene on 7p, the transforming growth factor gene on chromosome 2p, and the c-myc oncogene on chromosome 8q. However, it is likely that many of the genes involved in the initiation and progression of renal cancer are currently unknown. (7)

Specifically, loss of the short arm of chromosome 3 represents the main genetic aberration in ccRCC. At least three separate regions on 3p are involved in clear cell RCC. One is the von Hippel-Lindau disease (VHL) gene at 3p25–26, and others at 3p13–14, and 3p21. (8,9)

The von Hippel-Lindau syndrome is an autosomal dominant disorder characterized by the development of bilateral ccRCC, pheochromocytomas, hemangioblastomas of the central

nervous system, retinal angiomas, pancreatic cysts. Allelic losses at the 3p.25, where VHL gene is located, have also been shown to be particularly frequent in sporadic ccRCC. (10)

The VHL protein is involved in cell cycle regulation and angiogenesis. In particular, the loss of functional VHL suppressor gene results in loss of VHL-E3 ligase, a protein that can trigger a breakdown of the ubiquitous transcription factor HIF (Hypoxia-inducible factor); HIF in physiological conditions regulates the degradation of hypoxia inducible genes such as Vascular Endothelial Growth Factor (VEGF), platelet-derived growth factor (PDGF), the Epidermal Growth Factor (EGFR), the Transforming Growth Factor (TGF), and erythropoietin (EPO). Therefore, the damage of the VHL gene promotes an overexpression of these genes and an overproduction of their transcripts, resulting in neo-angiogenesis. For this reason, these molecules could be actually considered a potential target for therapies with antiangiogenic properties. (11-13)

Multiple cytogenetic studies established that loss of DNA sequences on the short arm of chromosome 3 (3p, 3p13-pter) has to be considered as one of the primary events in the development of ccRCC, and apparently not involved in tumor progression. (8)

Therefore, while some genetic markers have been extensively confirmed as having prognostic utility, there are only few studies in which 3p deletions are considered as a potential prognostic marker in ccRCC. (14,15)

The understanding of the pathogenesis and progression of cancer has been greatly implemented by the increased application of new techniques of molecular biology. (16) Recently, a significant number of novel markers, which may have diagnostic and prognostic significance, have been proposed. Validation of these markers in multiple clinical specimens is currently performed by traditional histopathological techniques, which are time consuming, labour intensive and, therefore, economically costly.

The tissue microarray (TMA) is a new histopathologic technique, which allows to perform specific molecular-biologic analyses, to properly solve these problems. In 1986, Hector Battifora described a original method, which appeared to confer the most significant advantage of simultaneously examining several tissue samples under the same conditions, with the acquisition of large quantities of data (17,18).

The tissue microarray (TMA) would allow the rapid and cost effective validation of novel markers in multiple pathological tissue specimens, since tissues from up to a 1000 histology blocks can be arrayed accurately into a newly created paraffin block, at designated locations. Thus, TMA can significantly accelerate the processing of a very large number of tissue specimens with excellent quality, good reliability and preservation of original tissue. (19)

Though the large majority of TMA products have been usually utilized for immunohistochemical analyses, TMAs seem to be very promising also for the application of in situ hybridization techniques, including FISH analyses.

FISH (Fluorescence In Situ Hybridization) is a technique that combines fluorescence microscopy with conventional methods of molecular hybridization in situ. Deletions, translocations and amplifications are the main molecular alterations that can be viewed with the FISH technique.

The extensive application of FISH on archive formalin fixed, paraffin embedded tumor samples has allowed to identify new diagnostic and prognostic genetic markers. Several researchers have shown the feasibility of FISH methodology in identifying genetic abnormalities in RCC.

Aim of our study is to investigate the gene status of 3p in 122 ccRCC using tissue microarrays with FISH and correlate this data with follow up of the patients.

MATERIALS AND METHODS

One-hundred and twenty-two patients with histopathologic diagnosis of ccRCC were retrieved for this study.

Formalin-fixed, paraffin-embedded tumor specimens were selected from radical or partial nephrectomies collected at the Department of Pathology of Verona University.

A critical review of the 122 ccRCC cases included in the study was performed, to define Fuhrman grade and TNM stage, according to the most recent classification criteria, namely, the size and the extension of the tumor, as defined by the 2002 TNM staging system proposed jointly by the Union of International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC) (4).

Construction and Sectioning of Tissue Microarrays.

Tissues from 122 histology paraffin blocks have been accurately arrayed into newly created 13 paraffin blocks.

We have identified the notebook donor and the area to select. After selection of the TMA-support of suitable dimension we have selected the tip of the corresponding TMA. Approaching the tip of the gun-layer in the notebook, in correspondence of the selected area, we have pressed still complete penetration in the notebook. To this point the tip from the extracted notebook becomes part in the hole correspondent of the selected TMA-support releasing the extracted tissue one delicately, in such way that the notebook is uniform for the cut.

It is necessary to use a map in order to recognize the champions of tissue inserted in every hole. Once filled up all the TMA-support with several, 1.5 mm in diameter, cylinders

captured from original paraffin blocks, we inserted it in the mold of inclusion, turning the cut surface towards the low, obtaining new TMA paraffin blocks.

Paraffin blocks were representative of at least three different areas of the neoplastic areas, and at least two from non-neoplastic renal parenchyma. Four μm sections were obtained by paraffin blocks. The sections have been subsequently stained with hematoxylin and eosin.

Immunohistochemical analysis

Additional consecutive slides have been utilized for immunohistochemical staining.

Slides were deparaffinized twice in xylene for 5 min and rehydrated through graded ethanol solutions to distilled water. Antigen retrieval was performed by heating sections in citrate buffer (AE1/AE3, CD10) or enzymatically with proteinase K (CAM5.2 and CK7).

No pretreatment was utilized for vimentin immunostaining.

Inactivation of endogenous peroxidase activity was obtained by incubating sections in 3% H2O2 for 15 min. Localization of bound antibodies was performed with peroxidase labelled streptavidin–biotin system with 3,30-diaminobenzidine as a chromogen.

Appropriate positive controls for each antibody were run concurrently and showed adequate immunostaining.

FISH analysis

Three μm sections from each block were cut for FISH analyses; the slides were deparaffinized with xylene, and subsequently washed twice with absolute ethanol. The slides then were air dried in the hood. Next, the slides were treated in 0.1mM citric acid (pH6.0) at 951C for 10 min, rinsed in distilled water for 3 min, and followed by a wash of 2XSSC (standard saline citrate) for 5 min. Digestion of the tissue was performed by applying 0.4 ml of pepsin (5 mg/ml in 0.9% NaCl, pH 1.5) at 37°C for 40 min. The slides were rinsed with distilled water for 3 min and further washed with 2XSSC for 5 min, and then allowed to air dry. FISH was performed with a mixture of centromeric α-satellite DNA probes for chromosome 3 (CEP3, Spectrum Orange) and subtelomeric probes for 3p25 (3pTel25, Spectrum Green).

The probes were diluted with t-DenHyb2 in a ratio of 1:100. About 5 μl of diluted probe were applied under conditions of reduced lighting on each section that was then covered with cover-glass object carefully sealed with mastic. He then proceeded to the denaturation and hybridization, which were obtained by using a wet room thermostat and timer (Hybrite) by incubating sections at 83 ° C for 12 minutes and returned to 37 ° C overnight. At this point after removing the cover-object and always in low lighting conditions, have been performed post-hybridisation washes with solutions warmed in an oven at 45 ° C: two consecutive washes in 0.1SSC/1.5M Urea, for 20 minutes each , 1 2XSSC washing for 20 minutes and a final wash of 10 minutes in 2XSSC/0.1% NP40. We then made a final wash at room temperature for 5 minutes 2XSSC. Without drying sections, we applied the fluorescent chromogenic substrate, 4,5-diamino-2-phenyl-indole

(DAPI), and the cover-object, sealing it with plenty of nail polish. Slides were examined using an Olympus fluorescence microscope equipped with selective filters for DAPI and the Spectrum Green and Spectrum Orange signals.

For each tumor, at least 100 nuclei from the 2 neoplastic cores were scored for signals from centromeric probes under the fluorescence microscope with X1000 magnification. At least 50 nuclei from non-neoplastic renal cores were also scored as control. Signals from solitary nuclei were preferentially counted, but signals from adjacent, non-overlapping nuclei were occasionally included in the counts. Overlapping nuclei and nuclei with uncertain signals were not included in the counts.

To evaluate the presence of 3p deletion, we scored the number of nuclear signals as single, double and 3 or more, for both subtelomeric and centromeric probes, expressed in percentages on the whole number of examined nuclei. Furthermore, we considered as 3p deleted all the neoplasms with ratios 3p/CEP3 below 0.7, as previously reported.

We have obtained follow-up data from the vast majority of patients, with the help of the colleagues in the departments of Oncology and Urology.

RESULTS

Revision and follow up data:

The age of patients ranged from 30 to 86 years with an average age of about 60 years, 31 patients were female, 91 male.

Such tumours were characterized by a variable alveolar, acinar or solid architectural pattern. All tumours contained a prominent, fine and delicate network of small thin-walled blood vessels. Areas of cystic degeneration and haemorrhage were frequent. The cellular population showed abundant and clear cytoplasm and well distinct cellular membranes.

The grading of the 122 cases included in the study was distributed in: Furhman grade 1 in 6 cases, grade 2 in 52 cases, grade 3 in 52 cases and grade 4 in 12 cases.

The tumor size ranged from 2 cm to 19 cm.

The TNM staging at diagnosis showed an uneven distribution of cases with a slight predominance of intermediate stages of the disease, namely 57 cases with pT1 stage, 25 with pT2,10 with pT3a, 25 with pT3b, and 5 with pT4.

Lymph node involvement at diagnosis was observed in 5 cases, respectively 1 case with pT3a stage, 3 with pT3b, 1 with pT4; no lymph node metastases were reported in pT1 and pT2 cases.

Metastatic spread at diagnosis was found in 17 of the 122 cases examined, respectively 2 cases with pT1 stage, 2 with pT2, 3 with pT3a, 8 with pT3b, and 2 with pT4.

Clinico-pathologic data are summarized in Table 1.

Follow-up data were available for all 122 selected patients: 36 patients died of disease, 8 patients died for unrelated causes, 77 patients were alive with no evidence of disease, and 1 patient was alive with disease progression. The follow-up data are based on a minimum of 2 months to a maximum of 168 months.

The immunoprofile of the tumours were typical of the clear cells variants. (CD10+, Vimentin+, low-molecular-weight cytokeratins+, CAM5.2+ and AE1/AE3+).

FISH analyses

FISH analyses were performed on 13 TMA slides, accounting for 122 different ccRCC with different grades and stages.

According to the criteria exposed in the Methods section, related to the number of nuclei to be scored for each tumor sample, informative cases were limited to 110. For the remaining 12 cases, we could not reach the adequate number of nuclei to evaluate for centromeric and locus-specific signals, due to the presence of focal hemorrhagic or necrotic areas; nevertheless, the number of nuclei which could be evaluated in these cases ranged from 60 to 85. Conversely, all the non neoplastic renal samples could be scored, due to the integrity and the well represented tubular component of kidney parenchyma.

Among the110 evaluable cases, we could be able to identify 3p losses in 78 tumor cases (71%), with ratios 3p/CEP3 ranging from 0.46 to 0.68 (mean: 0.55); the remaining 32 cases (29%) showed ratios ranging from 0.72 to 1.1 (mean: 0.92), and were considered to be negative.

The analysis of non neoplastic renal parenchyma showed ratios 3p/CEP3 consistently above the cut-off value of 0.7, ranging from 0.95 to 1.2.

Correlating the results of the FISH analysis for 3p25 gene status with stage, cases showing 3p deletion were predominantly low stage, with 78% included in pT1-T2 categories, whereas 22% were high stage, included in pT3-T4 categories; conversely, non deleted cases were mostly high stage (66%) with only one third low stage (34%) [Tables: 2,3,4].

With regard to the correlation between Fuhrman grade and 3p deletion, we could not find significant variations in the distribution of grade; in contrast, non deleted cases showed a prevalence of high grade (g3: 47%, g4: 25%). [Tables: 5,6,7].

About patients' follow-up, from among the 78 cases with 3p deletion, 62% were alive with no evidence of disease, compared with only 14% who died of disease. For the 32 cases without 3p deletion, 71% died of cancer, and 21% are alive with no evidence of disease. Out of a total of 110 cases evaluated, 56% of patients who were alive with no evidence of disease, showed deletion of 3p, whereas 20% of patients who died of disease had no evidence of 3p deletion. [Tables 8,9,10,11].

Finally, the 16 cases with metastatic disease, 5 of which with lymph node involvement, 6 cases showed 3p deletion, while 10 cases had no deletion.

Table 1: Clinical and pathological features

CASE	Age	Sex	Grade	Dimension (cm)	pTNM
1	52	m	g3	16	pT2mNoM1
2	86	f	g2	10	pT3bNoMo
3	58	m	g2	7	pT1NoMo
4	62	m	g3	3	pT1NoMo
5	61	m	g1	8	pT2NoMo
6	74	m	g3	7	pT1NoMo
7	60	m	g3	5	pT3bNoM1
8	81	m	g3	4	pT1NoMo
9	38	m	g2	5,5	pT1NoMo
10	55	f	g3	10,5	pT3bNoM1
11	73	m	g2	3,5	pT1NoMo
12	56	m	g2	4,5	pT1NoMo
13	72	m	g3	6	pT3aNoM1
14	64	m	g2	6,4	pT1NoMo
15	65	m	g3	8,5	pT3aNxMo
16	45	m	g3	6	pT1NoM1
17	68	m	g4	9	pT2NoMo
18	56	m	g2	4	pT1NoMo
19	65	f	g3	8	pT2NxMo
20	54	m	g2	3,5	pT1NoMo
21	68	f	g2	5	pT3bNoMo
22	64	m	g2	6	pT3bNoMo
23	60	m	g3	5	pT1NoMo
24	73	m	g3	6	pT1NoMo
25	55	m	g3	6	pT3aNoMo
26	65	m	g3	12	pT1NoMo
27	81	m	g3	7	pT2NoMo
28	66	f	g2	12	pT2NoMo
29	65	f	g4	4,5	pT2NxMo
30	70	m	g4	19	pT2mNoMo
31	43	m	g2	7	pT3bNoMo
32	58	f	g3	7	pT3bNoMo
33	36	m	g2	13	pT3bNoMo
34	63	m	g3	10	pT2NoMo
35	53	m	g1	4	pT1NoMo
36	44	m	g3	7,5	pT2NoMo
37	47	m	g4	12	pT3bNoMo
38	70	f	g2	5	pT1NoMo
39	45	m	g4	11,5	pT2NoM1
40	65	f	g2	5	pT1NoMo
41	62	m	g3	5	pT1NoMo
42	62	m	g3	6	pT3bNoM1
43	66	f	g1	3,5	pT1NoMo
44	75	m	g2	5	pT1mNoMo
45	62	m	g3	3,5	pT1NoMo
46	41	m	g2	8	pT2NoMo
47		f	g2	5	pT1NoMo

CASE	Age	Sex	grade	Dimension (cm)	pTNM
48	47	f	g3	3	pT1NoMo
49	79	f	g3	7,5	pT3bNoMo
50	46	m	g2	7	pT3bNoMo
51	67	f	g3	5	pT1NoMo
52	62	f	g2	2,5	pT1NoMo
53	56	m	g3	6	pT4NoMo
54	51	m	g3	12	pT2NoMo
55	73	m	g3	4	pT3bNoMo
56	53	m	g3	8	pT2NoMo
57		f	g2	6	pT4NoM1
58	50	m	g2	8,5	pT2NoMo
59	80	m	g2	10	pT2NoMo
60		m	g2	6	pT3bNoM1
61	41	f	g2	2	pT1NoMo
62	76	m	g3	9,5	pT3bN1M1
63	71	m	g2	6,5	pT1NoMo
64	65	m	g3	14	pT2NoMo
65	49	m	g3	13	pT2NoMo
66	31	m	g2	4	pT1NoMo
67	71	m	g3	11	pT3bNoMo
68	68	m	g2	3,5	pT3aN2M1
69	72	m	g2	2,5	pT1NoMo
70	53	f	g2	4	pT1NoMo
71		m	g2	9	pT4N1M1
72	53	m	g2	6	pT3aNoMo
73	48	f	g4	8	pT2NoMo
74	53	m	g3	6	pT1NoMo
75	60	m	g3	12	pT4NoMo
76	64	m	g3	6	pT1NoMo
77	49	m	g2	3,5	pT1NoMo
78	54	f	g2	8	pT3bNoMo
79	65	f	g2	4	pT1NoMo
80	50	f	g4	18	pT3aNoMo
81	69	m	g2	3	pT3bNoMo
82	61	m	g3	5	pT1NoMo
83	30	f	g2	5	pT1NoMo
84	59	m	g3	7	pT2mNoMo
85	44	m	g1	3,5	pT1NoMo
86	55	m	g4	7	pT3bN2M1
87	78	m	g3	6	pT3bNoMo
88	72	m	g4	10	pT3bN2M1
89	57	f	g1	8	pT2NoMo
90	72	m	g2	4	pT1NoMo
91	60	m	g2	9	pT2NoMo
92	54	f	g2	3,5	pT1NoMo
93	57	m	g2	8	pT2NoMo
94	57	m	g3	5,5	pT1NoMo
95	69	m	g3	5	pT1NoMo
96	57	m	g3	2,7	pT3aNoMo
97	61	m	g2	12,5	pT3bNoMo
98	45	f	g3	13	pT3aNoMo
99	60	m	g3	4	pT1NoMo

CASE	Age	Sex	grade	Dimension (cm)	pTNM
100	65	m	g1	3	pT1NoMo
101	67	f	g2	6,5	pT1NoMo
102	48	m	g2	3	pT1NoMo
103	55	m	g3	4	pT1NoMo
104	74	m	g4	4	pT1NoMo
105	46	m	g3	7	pT2NoMo
106	79	m	g4	6,5	pT3bNoMo
107	59	m	g2	5	pT1NoM1
108	67	m	g4	5	pT1NoMo
109	50	m	g2	2	pT1NoMo
110	79	m	g3	7,5	pT3bNoMo
111	63	m	g3	5,5	pT1NoMo
112	58	m	g2	3	pT1mNoMo
113	71	f	g2	6,5	pT2NoMo
114	78	m	g3	5,6	pT3bNoM1
115	61	m	g3	6	pT1NoMo
116	62	f	g2	9	pT3aNoM1
117	66	m	g3	11	pT4NoMo
118	71	m	g3	5,5	pT3aNoMo
119	57	f	g2	3,5	pT1NoMo
120	66	m	g2	6	pT1NoMo
121	63	m	g2	7	pT1NoMo
122	66	f	g3	7	pT1NoMo

Table 2: FISH analysis and stage: deleted cases

stage	n°	%
pT1	46	59%
pT2	15	19,00%
pT3	16	21,00%
pT4	1	1%
tot	78	100%

Table 3: FISH analysis and stage: not deleted cases

stage	n°	%
pT1	4	13%
pT2	7	21,00%
pT3	17	53,00%
pT4	4	13%
tot	32	100%

Table 4: FISH analysis and stage: not deleted and deleted cases

3p Status	Stage	n°	%
N.D.	pT1/2	11	10%
N.D.	pT3/4	21	19,00%
D	pT1/2	61	56%
D	pT3/4	17	15%
tot		110	100%

Legend: D: deletion; ND: no deletion

Table 5: FISH analysis and grade: deleted cases

Grade	n°	%
g1	4	5%
g2	38	49%
g3	32	41%
g4	4	5%
tot	78	100%

Table 6: FISH analysis and grade: not deleted cases

Grade	n°	%
g1	0	0%
g2	9	28%
g3	15	47%
g4	8	25%
tot	32	100%

Table 7: FISH analysis and grade: not deleted and deleted cases

3p Status	Grade	n°	%
N.D.	g1/g2	9	8%
N.D.	g3/g4	23	21,00%
D	g1/g2	42	37%
D	g3/g4	38	34%
tot		110	100%

Legend: D: deletion; ND: no deletion

Table 8: FISH analysis and follow up

CASE	Follow-up	Outcome	3p/CEP3	3p status
1	25	DOD	0,55	D
2	24	DOD	1,1	ND
3	145	ANED	0,66	D
4	151	ANED	0,91	ND
5	77	ANED	-	-
6	54	DOD	0,51	D
7	16	DOD	0,96	ND
8	58	ANED	-	-
9	86	ANED	0,51	D
10	14	DOD	0,97	ND
11	129	ANED	0,51	D
12	79	ANED	0,93	ND
13	58	DOD	0,91	ND
14	91	ANED	0,53	D
15	54	DOD	0,91	ND
16	32	DOD	0,8	ND
17	36	DOD	0,95	ND
18	67	ANED	0,54	D
19	118	ANED	0,97	ND
20	82	ANED	0,55	D
21	77	DOD	0,91	ND
22	134	ANED	0,65	D
23	126	ANED	0,55	D
24	54	ANED	0,65	D
25	36	DOD	0,93	ND
26	60	ANED	0,48	D
27	24	DOC	0,52	D
28	96	ANED	-	-
29	116	ANED	0,65	D
30	82	ANED	0,97	ND
31	48	ANED	0,65	D
32	32	DOD	0,48	D
33	55	ANED	0,65	D
34	107	ANED	0,65	D
35	134	ANED	0,46	D
36	104	ANED	0,68	D
37	34	DOD	0,9	ND
38	81	ANED	0,56	D
39	7	DOD	0,93	ND
40	131	ANED	0,66	D
41	145	ANED	0,65	D
42	12	DOD	-	-
43	60	ANED	0,65	D
44	152	DOC	0,47	D
45	162	ANED	-	-
46	120	ANED	0,5	D
47	49	ANED	0,48	D

CASE	Follow-up	Outcome	3p/CEP3	3p status
48	103	ANED	0,5	D
49	24	DOD	0,5	D
50	58	ANED	0,66	D
51	60	ANED	0,54	D
52	62	ANED	0,48	D
53	12	DOD	0,93	ND
54	96	ANED	0,8	ND
55	53	DOD	0,5	D
56	58	ANED	0,47	D
57	12	DOD	0,93	ND
58	98	ANED	0,51	D
59	55	ANED	0,56	D
60	64	DOD	0,65	D
61	111	ANED	0,48	D
62	12	DOD	0,93	ND
63	60	ANED	0,48	D
64	23	DOD	0,47	D
65	168	ANED	0,47	D
66	103	ANED	0,5	D
67	12	DOD	-	-
68	12	DOD	0,65	D
69	87	DOD	1,1	ND
70	132	ANED	0,51	D
71	3	DOD	0,98	ND
72	108	DOC	0,51	D
73	7	DOC	0,97	ND
74	80	ANED	0,51	D
75	60	DOD	0,82	ND
76	48	ANED	0,51	D
77	103	ANED	-	-
78	108	ANED	0,85	ND
79	62	ANED	-	-
80	69	DOD	0,93	ND
81	43	DOD	0,8	ND
82	127	DOD	0,65	D
83	96	ANED	-	-
84	84	ANED	0,51	D
85	140	ANED	0,51	D
86	12	DOD	0,91	ND
87	134	DOC	0,93	ND
88	2	DOD	0,72	ND
89	160	ANED	-	-
90	60	ANED	0,53	D
91	74	AWD	0,54	D
92	142	ANED	0,48	D
93	113	ANED	0,6	D
94	49	ANED	-	-
95	86	ANED	0,65	D
96	119	DOD	0,65	D
97	56	DOD	0,9	ND
98	84	ANED	0,65	D

CASE	Follow-up	Outcome	3p/CEP3	3p status
99	61	ANED	0,48	D
100	154	ANED	0,49	D
101	142	ANED	0,6	D
102	114	ANED	0,65	D
103	100	ANED	0,5	D
104	20	DOC	0,49	D
105	116	DOD	0,97	ND
106	24	ANED	0,5	D
107	55	ANED	0,48	D
108	47	DOC	0,49	D
109	153	ANED	0,49	D
110	129	ANED	0,93	ND
111	63	ANED	0,49	D
112	97	ANED	0,47	D
113	48	ANED	0,48	D
114	64	ANED	0,65	D
115	24	DOD	0,51	D
116	52	ANED	0,49	D
117	110	ANED	0,65	D
118	49	ANED	0,65	D
119	136	DOC	-	-
120	129	ANED	0,65	D
121	129	ANED	0,53	D
122	114	ANED	0,5	D

Legend: D: deletion; ND: no deletion; DOD: dead of disease; ANED: alive with no evidence of disease; AWD: alive with disease; DOC: dead of unrelated causes;

Table 9: FISH analysis and follow up: delete cases

Outcome	n°	%
DOD	11	14%
DOC	5	6,40%
ANED	62	79,40%
tot	78	100%

Legend: DOD: dead of disease; ANED: alive with no evidence of disease; DOC: dead of unrelated causes

Table 10: FISH analysis and follow up: no delete cases

Outcome	n°	%
DOD	23	71%
ANED	7	21%
DOC	2	8%
tot	32	100%

Legend: DOD: dead of disease; ANED: alive with no evidence of disease, DOC: dead of unrelated causes

Table 11: FISH analysis and follow up N.D and D. cases

3p Status	Outcome	n°	%
N.D.	DOD	23	20%
N.D.	ANED	7	6,40%
N.D.	DOC	2	1,80%
D	DOD	11	11%
D	DOC	5	5%
D	ANED	62	56%
tot		110	100%

Legend: D: deletion; ND: no deletion; DOD: dead of disease; ANED: alive with no evidence of disease,

DISCUSSION

Renal cell carcinomas are heterogeneous tumors that include several distinct entities with a range of biological and clinical behaviors, from relatively favorable to extremely aggressive. The heterogeneity leads to unpredictable outcome and survival.

Several pathological parameters, including stage, grade and histologic type have been associated with prognosis in renal cell carcinoma. Specifically, Fuhrman nuclear grade has been shown to be an independent predictive factor of survival in many studies, higher grades correlating with the biological aggressiveness of the tumor and increased metastatic potential.(20-23)

Recently, studies using current histological subtyping of RCC based on the UICC/AJCC and Heidelberg recommendations from 1997(24) or the similar WHO histological classification from 2004, have identified histology as an important prognostic factor of RCC. (4,25-26)

These classifications include the following distinct malignant histological subtypes: clear cell RCC, papillary RCC, chromophobe RCC, collecting duct RCC, and unclassified RCC. Oncocytoma and metanephric adenoma are classified as benign tumors. Each of the malignant histological subtypes is associated with distinct cytogenetic abnormalities and prognostic differences, affecting both the metastatic potential of the tumors and survival of the patients. Indeed, clear-cell renal cell carcinoma shows a significantly poorer cancer-specific survival than patients with papillary or chromophobe.

Although pathological parameters of RCC provide important prognostic information, in many cases they are insufficient to predict the clinical behaviour of these tumours.

RCC is well recognized as a malignancy with an unpredictable course. Some patients with comparable or the same histological tumours can show a wide variation in biological behaviour and clinical outcome. Moreover, individual patients with a different tumor stage and/or grade may have a similar survival. (27)

Indeed, pathological parameters do not take into consideration the molecular and biological features of the tumor. For this reason, new biologic markers are needed that could identify prognostic categories in renal tumors with the same pathological features and possibly aid in the selection of patients for postoperative treatment.

Recently, biologic factors, including nuclear proliferating index, angiogenesis, apoptosis, growth factors, and adhesion molecules, and their potential as prognostic markers have been studied in renal cell carcinoma. (28-34)

Moreover, studies characterizing genetic aberrations in RCC have implicated a number of chromosomal loci as important in cancer development and progression.

One of the most frequent aberration found in renal cancer is loss of the short arm of chromosome 3, which represents the hallmark genetic aberration detected in ccRCC.

Siebert et al. found loss of 3p in 15 of 19 clear cell RCCs and not in other subtypes.(35)

Presti at al. revealed a high percentage of chromosomal losses in 60/64 (94%) clear cell renal carcinoma by using two probes mapping for chromosome 3p24 and 3p25.(36)

Yoshiro at al detected deletions of 3p25 in 38/50 (76%), monosomy of chromosome 3 in 13/50 (26%) and gain of chromosome 3 (polisomy) in 10/50 (20%) cases of clear cell renal cell carcinoma. In this study both a centromeric chromosome 3 and locus specific 3p probes have been utilized.(37)

Nagao et al. Examined 50 RCCs by dual-color FISH with DNA probes for centromere 3 and for the locus 3p25.1, approximately p25.3. They found in 40/50 (80%) loss of LSI 3p. (38).

Moch et al. studied 53 CCRCCs using dual-color FISH with probes for the VHL gene and the chromosome 3 centromere. Deletion was detected in 69% of clear cell RCCs. (39).

Our study seems to confirm this genetic abnormality as a specific marker for this subgroup of renal cancers, and our results are in keeping with previous experiences in the analysis of 3p losses in ccRCC, showing 3p deletion in 71% of analyzed tumors.

However, 3p deletion, known as an early event involved in tumor initiation, rather than in tumor progression, has rarely been taken into consideration as predictive for clinical outcome. (8)

In this study, we also found that loss of 3p was associated with lower pT stage, lower risk of lymph node and distant metastases and low-grade tumors; we also observed a slight tendency towards an improved survival in 3p deleted cases, compared with those without 3p loss, and decreased risk of death for patients with loss of 3p material.

Our experience is in agreement with previous observations. Klatt et al. observed that VHL gene inactivation has been linked with better prognosis. They observed in 282 patients affected by ccRCC that 3p/VHL losses were associated with improved survival and decreased risk of death (14).

Likewise, Parker et al. demonstrated in a series of 273 ccRCC that the absence of pVHL (VHL protein) detected by immunohistochemistry was associated with improved survival. (15). However, since both loss of 3p and VHL inactivation were correlated with TNM stage and grade, they were not retained as independent prognostic factors in a multivariate analysis.

An analysis of a larger number of cases and further molecular studies will be necessary to fully characterize ccRCC with same morphology and immunophenotype but different clinical outcome.

Tissue microarray technology allows rapid visualization of molecular targets in thousands of tissue specimens. TMAs are ideally suitable for genomic based diagnostic and drug target discovery. The speed of molecular analyses is increased by more than 100-fold, precious tissues are not destroyed and a very large number of molecular target can be

analyzed from consecutive TMA sections. Most of the application of the TMA technology have come from the field of cancer research.

The frequencies of molecular alterations found by TMA analysis correspond very well with the published frequencies derived from studies with conventional tissue samples. The validity of TMA analysis has been shown by comparisons with whole-section analysis in breast (40,41), prostate (42,43), and brain cancer (44). The only limitation in TMA application could be related to the well known tumor heterogeneity observed in RCC, which could be responsible for some of the negative cases in our study. Besides, in our study the non informative cases, which accounted for 7.5% of the tumors, have been related to the presence of unexpected small necrotic and/or hemorrhagic foci that could emerge in consecutive tissue sections.

The correlation of data derived from TMA with clinical follow-up, in formulating or evaluating the prognosis of the patient, is of great interest at the clinical level. A good example of the prognostic value of TMA application is represented by Varambally et al. experience on protein EZH2 expression on prostate cancer (45). Similar associations with prognosis have been described in breast (46), bladder (47) and kidney cancers (48), using arrays prognosis.

FISH technique is ideally applicable to the analysis of genetic alterations on TMA slides. A single hybridization provides visualization of specific genetic changes in up to 1000 tissues.

Brunelli et al. in 2009 demonstrated that tissue microarrays are a valid substitute for whole tissue sections in large cohort studies, when FISH analysis is undertaken. The study was performed to compare numerical chromosomal changes obtained from whole tissue sections with those of tissue microarrays in a series of chromophobe renal cell carcinoma. Concordance of results was improved when the number of analyzed cores was increased

from 2 to 3, at which point a concordance index ranging from substantial to almost perfect was observed. (49)

To date, only a few studies have been reported about TMA application on ccRCC.

Dahinden et al. evaluated by TMA the expression patterns of 15 different proteins in over 800 ccRCC patients, to analyze pathways reported to be physiologically controlled by the VHL tumor suppressor protein. (50)

In a study of Eckel-Passow et al. tumor expression of 6 biomarkers (B7-H1, B7-H3, survivin, Ki-67, CAIX, and IMP3) with variable expression patterns was evaluated in 100 patients with ccRCC, by means of TMA. (51)

The genetic profile of these tumors may aid in making the correct diagnosis, understanding the biologic mechanisms for tumor development and progression, accurately assessing prognosis, and selecting appropriate and targeted therapeutic options.

In summary, we analysed 3p deletions in a series of ccRCCs using FISH analyses in TMA and we have found that:

1) Loss of 3p has been confirmed as a genetic hallmark of ccRCC.

2) Loss of 3p was associated with lower pT stage, lower risk of lymph node and distant metastases and low Fuhrman grade tumors; we also observed a slight tendency towards an improved survival in 3p deleted cases.

3) TMA has been demonstrated as a reliable technique for FISH investigations on formalin fixed, paraffin embedded neoplastic specimens, since the considerable reduction in time consuming activities and the significantly lower cost of this procedure, that allows to reduce of more than 90% the amount of probes to be utilized.

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FIGURES

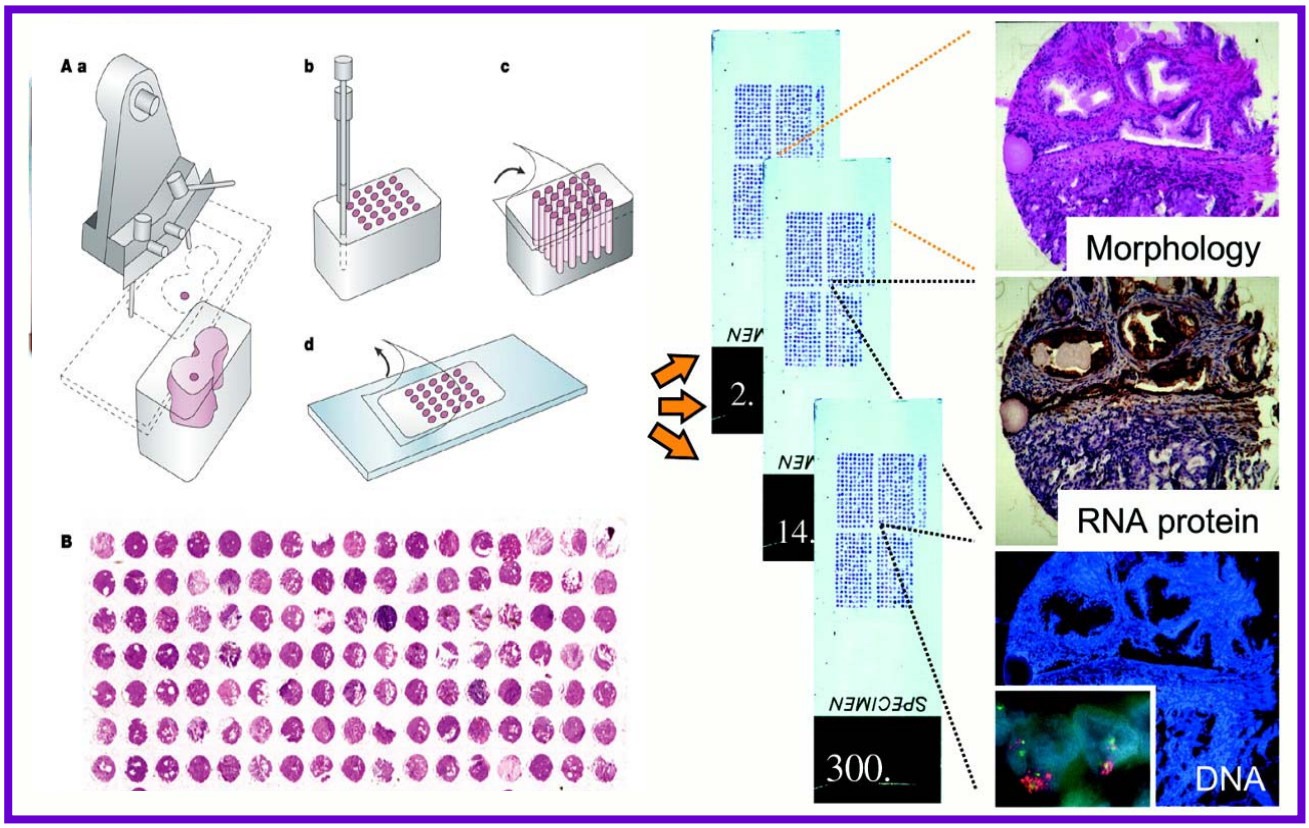

Fig.1:TMA construction and application

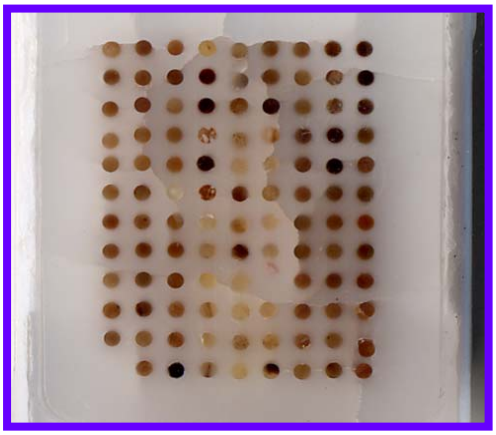

Fig.2:TMA newly created paraffin block from ccRCC specimens

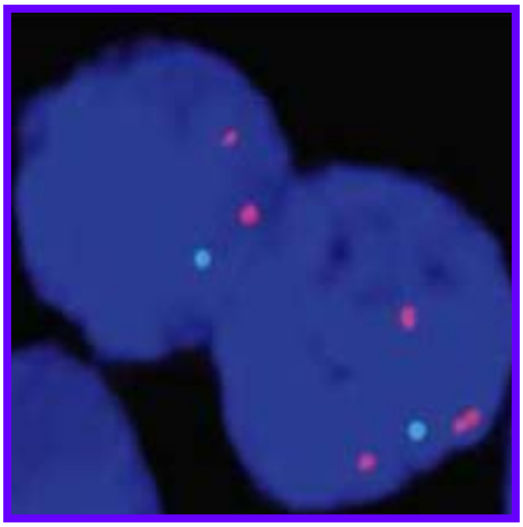

Fig. 3: FISH analysis of 3p: Deletion

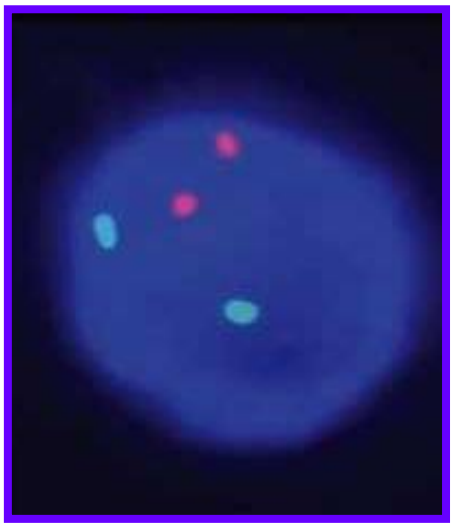

Fig. 4: FISH analysis of 3p: No Deletion

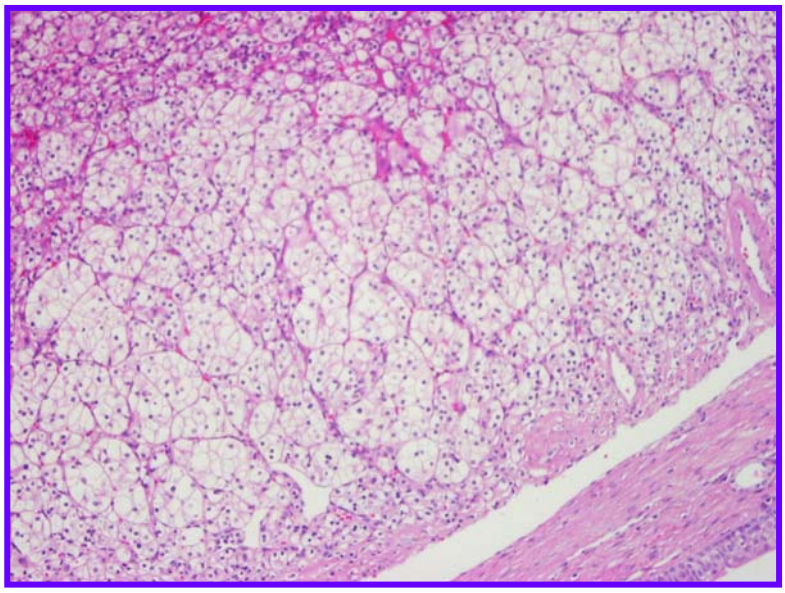

Fig.5 CCRCC Fuhrman Grade 1(H&E stain)

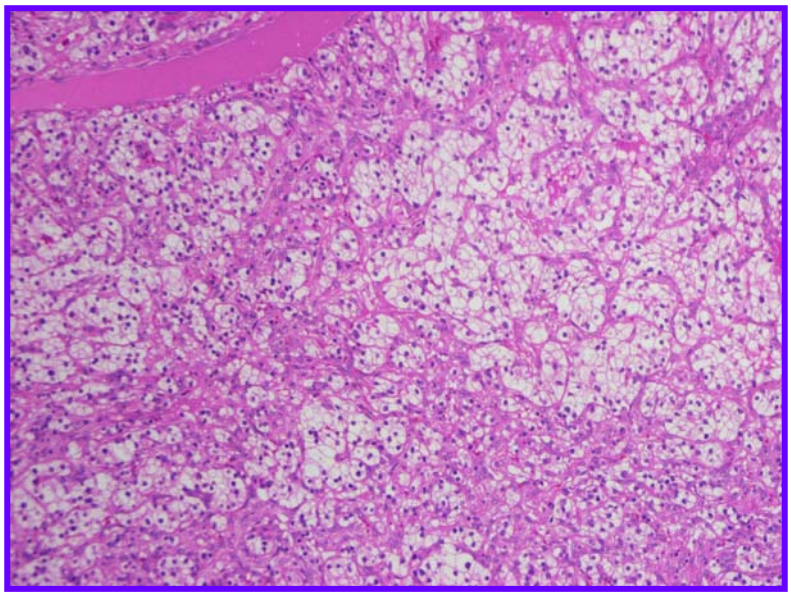

Fig.6 CCRCC Fuhrman Grade 2 (H&E stain)

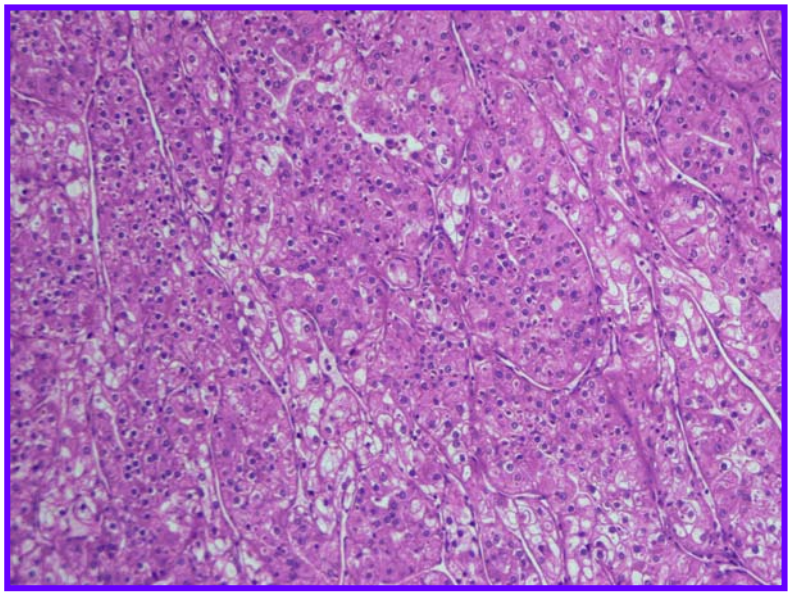

Fig.7 CCRCC Fuhrman Grade 3 (H&E stain)

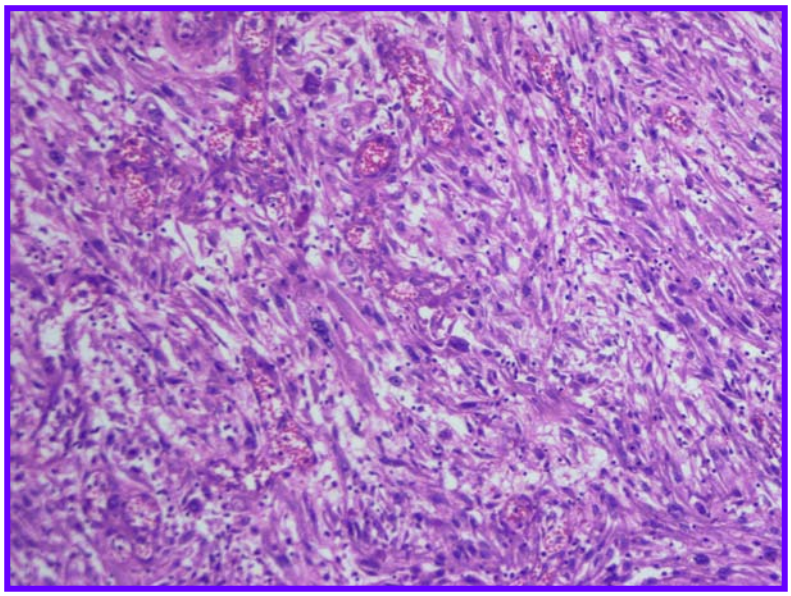

Fig.8 CCRCC Fuhrman Grade 4 sarcomatoid (H&E stain)