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IMMUNOHISTOCHEMICAL EXPRESSION OF TUMOR ANTIGENS MAGE-A3/4 AND  
NY-ESO-1 IN RENAL ONCOCYTOMA AND CHROMOPHOBE RENAL CELL  
CARCINOMA

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## SUMMARY

Distinction between renal oncocytoma (RO) and chromophobe renal cell carcinoma (ChRCC), especially the eosinophilic variant, can often be difficult. Our study has documented for the first time the expression of MAGE-A3/4 and NY-ESO-1 cancer testis antigens (CTAs) in these tumors. A total of 35 patients (17 ROs and 18 ChRCCs) were included in the study. Two antibodies were used for immunohistochemical staining: 57B recognizing multiple MAGE-A and D8.38 recognizing NY-ESO-1 CTAs. Fifteen (88.2%) samples of RO stained positively for both MAGE-A3/4 and NY-ESO-1 antigens. Regarding ChRCC, seven (38.9%) stained positively for MAGE-A3/4 and six (33.3%) for NY-ESO-1 antigens. Median MAGE-A3/4 expression was moderately positive in RO and negative in ChRCC. The difference in MAGE-A3/4 expression between two tumor groups was significant ( $P=0.0013$ ). Median NY-ESO-1 expression was strongly positive in RO and negative in ChRCC. The difference in NY-ESO-1 expression between two tumor groups was also significant ( $P=0.0008$ ). Our study has showed that RO had significantly higher expression of both CTAs. However, additional research is needed to clarify their potential diagnostic implications.

## INTRODUCTION

Renal oncocytoma (RO) and chromophobe renal cell carcinoma (ChRCC) are both renal epithelial neoplasms thought to arise from intercalated cells of collecting ducts. Together they account for approximately 10% of surgically removed renal epithelial tumors (4). Oncocytomas are benign, nonencapsulated neoplasms composed of round-to-polygonal cells with densely granular eosinophilic cytoplasm (so-called oncocytes), which form compact nests, acini, tubules or microcysts. Oncocytomas occasionally have sclerosed central area (4,14). ChRCC are solid tumors made up of large polygonal cells with prominent cell membranes, pale cytoplasm and usually a perinuclear halo. They include three subtypes: classic, eosinophilic and mixed (24). The majority of ChRCCs are stage T1 and T2 and only a few cases of lymph node and distant metastasis have been described (4).

Distinction between RO and ChRCC, especially its eosinophilic variant, can sometimes be difficult due to their overlapping morphological characteristics. Histology, ultrastructural examination and staining with Hale's colloidal iron can be used for their differentiation in daily practice. In recent years, there have been attempts to find an immunohistochemical marker that could also help in diagnostics (1,5,15-17,19).

Cancer testis antigens (CTAs) comprise a family of more than 40 genes expressed in a wide variety of malignant tumors (21). In normal tissue, their expression is mostly limited to germ cell lines. Because of their ability to induce immune responses, CTAs are being evaluated as targets for therapeutic cancer vaccines (3,23).

Few information are available on the expression of CTAs in different histological subtypes of renal tumors (18,25).

The aim of this study was to investigate the immunohistochemical expression of MAGE-A3/4 and NY-ESO-1 CTAs in ROs and ChRCCs. To our knowledge, there are no studies regarding immunohistochemical expression of these CTAs in RO and/or ChRCC.

## MATERIAL AND METHODS

### *TISSUE SAMPLES*

Pathology reports of histologically confirmed ROs and ChRCCs diagnosed at two Departments of Pathology (Ljudevit Jurak University Department of Pathology, Sestre milosrdnice University Hospital and Department of Pathology, University Hospital Dubrava, Zagreb) were reviewed. The diagnosis of all cases was established according to the criteria set forth in the WHO Classification of Tumors of the Urinary System and Male Genital Organs from 2004 (4). There were 35 cases in total: 17 ROs and 18 ChRCCs. Among patients with RO, 10 were females and 7 males. Patients' age ranged from 47-80 years (mean 64.4). Tumor size ranged from 0.9-8 cm (mean 3.7 cm). Among patients with ChRCC, 11 were females and 7 males. Patients' age ranged from 34-76 years (mean 58.2). Tumor size ranged from 1.7-17cm (mean 7.6).

### *IMMUNOHISTOCHEMISTRY*

Two antibodies were used for immunohistochemical staining. 57B was generated on immunization of mice with recombinant MAGE-A3 (13). However, this antibody recognizes a variety of MAGE-A molecules and it is currently considered a multi-MAGE-A-specific reagent (11). D8.38 antibody, recognizing NY-ESO-1 and its homologous LAGE-1 CTA, has been previously described (22).

Tissue sections of 3 to 5  $\mu$ m thickness were cut from paraffin-embedded tissue blocks, placed on object slides (Menzel-Glaser, Germany) and incubated for 20 minutes in a thermostat at 60°C.

The sections were then deparaffinized and incubated for 3 x 5 minutes in 10 mmol/L of citrate buffer (pH 6.0) in a microwave oven at 800 W. Subsequently, tissue slides were

washed with phosphate-buffered saline (PBS) buffer (pH 7.2), and endogenous peroxidase activity was blocked by a 5-minute treatment with hydrogen peroxide (Dako, No. S2023). Slides were then washed with PBS buffer and incubated for 90 minutes with MAGE-A3/4 57B or NY-ESO-1 D8.38 undiluted supernatants at room temperature.

After washing in PBS, the secondary biotinylated antibody (DAKO, No.K0690) was added for 30 minutes of incubation. Slides were then washed with PBS-buffer and treated with streptavidin-horseradish peroxidase (Dako-No.K0690) for 30 minutes. Tissue sections were washed once more in PBS-buffer and then Chromogen (Dako, No.K3468) was added for 5 minutes. Slides were washed in distilled water, stained with hemalaun (Dako, No.S2020) for 1 minute, washed with water, dehydrated with alcohol (96%), cleared with xylene, and mechanically covered.

Melanoma and testicular tissues expressing CTAs were used as positive controls. For negative control we replaced primary antibodies with isotype matched immunoglobulins.

The results of the immunohistochemical staining were expressed semiquantitatively as follows: negative response (-): no staining in tumor cells; weakly positive response (+): up to 10% of tumor cells positive; moderately positive response (++) : >10-50% of tumor cells positive; and strongly positive response (+++) : more than 50% of tumor cells positive.

#### *STATISTICAL ANALYSIS*

Statistical analysis was done using Mann-Whitney and Spearman's rank correlation tests.  $P < 0.05$  was considered to be statistically significant.

## RESULTS

Clinical and histological data and results of immunohistochemical staining are summarized in Tables 1 and 2. Fifteen ROs (88.2%) were positive for both MAGE-A3/4 and NY-ESO-1 antigens (Fig 1A and B). Regarding ChRCC, 7 (38.9%) showed positive reaction for MAGE-A3/4 antigen and 6 (33.3%) for NY-ESO-1 antigen (Fig 1C and D). Median MAGE-A3/4 expression was moderately positive in ROs and negative in ChRCCs. The difference in MAGE-A3/4 expression between two tumor groups was statistically significant ( $P=0.0013$ ). Median NY-ESO-1 expression was strongly positive in ROs and negative in ChRCCs. The difference in NY-ESO-1 expression between two tumor groups was also significant ( $P=0.0008$ ). The pattern of staining was diffuse cytoplasmic. Comparison of the expression of MAGE-A3/4 and NY-ESO1 antigens with the nuclear grade in ChRCC showed no statistically significant correlation ( $p=0.9$  and  $p=0.7$ , respectively). Also the size of ChRCC and the expression of MAGE-A3/4 and NY-ESO1 did not correlate significantly ( $p=0.4$  and  $p=0.6$ , respectively).

## DISCUSSION

Renal oncocytoma and ChRCC, especially its eosinophilic variant, can often be confused with one another due to their similar morphology. Distinction between these tumors is clinically relevant because they have different biological courses; RO being a benign neoplasm whereas ChRCC has malignant potential, particularly its sarcomatoid variant which is associated with more aggressive tumor behavior (4).

There have been numerous studies that explored the possible use of various immunohistochemical markers in differentiation of RO from ChRCC.

Garcia et al (5) pointed out the usefulness of caveolin-1 immunohistochemical analysis in differentiation of these tumors. In their study all 21 ChRCCs (100%) and only 3 (12%) of 26 ROs showed positive reaction for this marker (5).

Findings regarding kidney-specific cadherin remain contradictory. While some studies have showed that kidney-specific cadherin was almost exclusively expressed in ChRCC (16), other more recent studies strongly suggest that it cannot be used in differentiation of these tumors (1). Memeo et al (17) identified specific staining patterns of the 4 major histologic subtypes of renal neoplasms according to their reaction to cytokeratin 7 (CK 7), KIT and PAX 2. The predominant expression profile was CK7-/KIT+/PAX2+ for RO and CK7+/KIT+/PAX2- for ChCRR (17). Liu L et al (15) also found CK 7 to be positive in the majority of ChRCC (86%) but in none of ROs. In addition, they suggested that homogeneous epithelial cell adhesion molecule (EpCAM) expression confirms the diagnosis of ChRCC rather than RO (15). The latest markers considered are claudin-7 and 8. In the study by Osunkoya et al (19) claudin-7 protein was expressed in a membranous pattern in 4 of 17 ROs and 10 of 11 ChRCCs. Claudin-8 was expressed in a membranous or cytoplasmic pattern in 15 of 17 ROs and in a membranous pattern in 3 of 11 ChRCCs (19).



Several authors studied the genetic abnormalities in the subset of renal epithelial tumors, including ROs and ChRCCs. Brunelli et al (2) analyzed a group of 29 renal neoplasms (10 oncocytomas, 9 eosinophilic ChRCCs and 10 classic ChRCCs) by fluorescence in situ hybridization with a conclusion that loss of chromosomes 2, 6, 10 or 17 is a helpful marker of eosinophilic ChRCC (2). Other authors examined different groups of renal epithelial tumors by virtual karyotyping with single nucleotide polymorphisms (SNP) microarrays (7,12). In their studies the majority or all of ChRCCs showed losses affecting chromosomes 1, 2, 6, 10, 13 and 17 while ROs displayed complete or partial loss of chromosome 1 in the majority of cases (7,12). In the study of chromosomal changes in ChRCCs and ROs by Yusenko et al (26) loss of chromosomes 2, 10, 13, 17 and 21 were characteristic of ChRCCs (26). Thus, all these authors concluded that the use of cytogenetic analysis is a very valuable tool in distinguishing the two tumor types. A recent work of Petersson et al (20) showed that cases of hybrid oncocytic/chromophobe tumors of the kidney do exist, outside the Birt-Hogg-Dubé syndrome, and these tumors display some characteristic chromosomal changes (20).

CTAs are immunogenic proteins predominately expressed in gametogenic tissue and malignant tumors (3,23). Their expression in renal cell carcinoma was investigated only by few authors. In the study of Yamanaka et al (25) high incidence of plural MAGE genes was found in the cohort of 50 renal cell carcinomas suggesting their suitability for immunotherapy (25). One of the investigated genes (MAGE-4) was more frequently expressed in the clear cell subtype than in granular cell subtype of renal cell carcinomas (25). Neumann et al (18) analyzed the expression of CTAs in renal cell carcinoma by reverse transcription-polymerase chain reaction, but only two cases of ChRCC were present in their study (18). ChRCCs predominantly expressed tumor-associated antigen RAGE-1 whereas tumor-associated antigen PRAME was more frequently observed in clear cell subtype of renal cell carcinoma (18). A correlation between tumor-associated antigens expression and morphological subtype

of renal tumors was mentioned in this study (18). However, due to small number of cases of ChRCCs these data were inconclusive.

Scanlan et al (21) compared mRNA expression frequencies in various cancers obtained from numerous sources. According to expression of cancer testis (CT) genes, they divided tumors into high (bladder cancer, non-small cell lung cancer, and melanoma), moderate (breast and prostate cancer) and low (renal cell and colon cancer) CT gene expressers. In renal cell cancer only 3 of totally 33 examined CT genes (9%) had an expression frequency greater than 20% (21). NY-ESO-1 had an expression frequency of 9% (MAGE-A3/4 expression has not been studied). MAGE-A3/4 expression has been found in prostate cancer, squamous cell carcinoma of the penis and non-small cell lung cancer (6,8,9). The study by Jungbluth et al (10) has showed that 20-30% of lung cancers, bladder cancers and melanoma stained positive to NY-ESO-1 but there was no expression in colon and renal cell cancer. Moreover, they pointed out that there is a great variability in NY-ESO-1 expression in individual tumors, ranging from an infrequent homogeneous pattern of staining to highly heterogeneous antigen expression (10).

To date there have been no studies that comprehensively evaluated CTA expression in ROs and ChRCCs. Our results have documented for the first time the expression of MAGE-A3/4 and NY-ESO-1 CTAs in RO and ChRCC by immunohistochemistry. Both CTAs were expressed in RO and ChRCC. The partial overlap in expression between the tumor groups could be explained by some cases of sporadic hybrid oncocytic/chromophobe tumors, since they have been described outside Birt-Hogg-Dubé syndrome, but we did not investigate their possible presence in our series.

However, the expression of both CTAs was significantly higher in RO compared to ChRCC. Nevertheless, additional research is needed to clarify their potential diagnostic implications.

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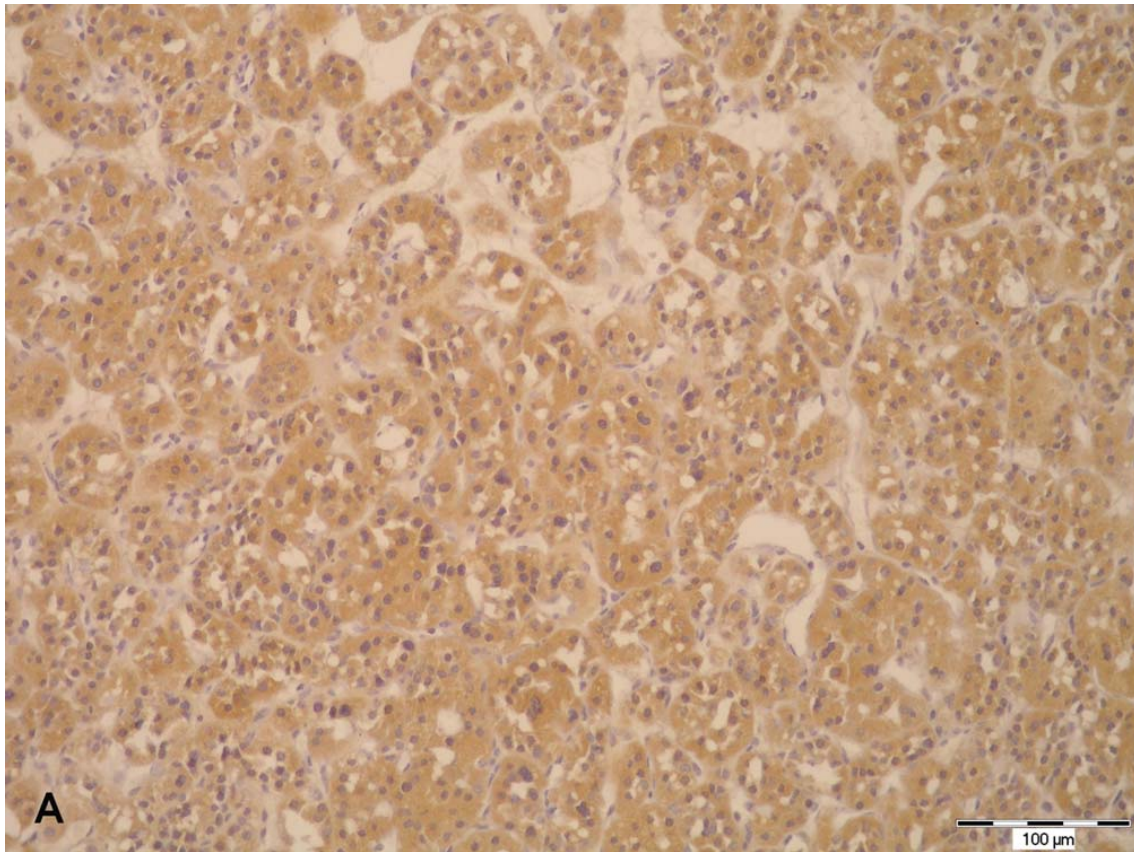
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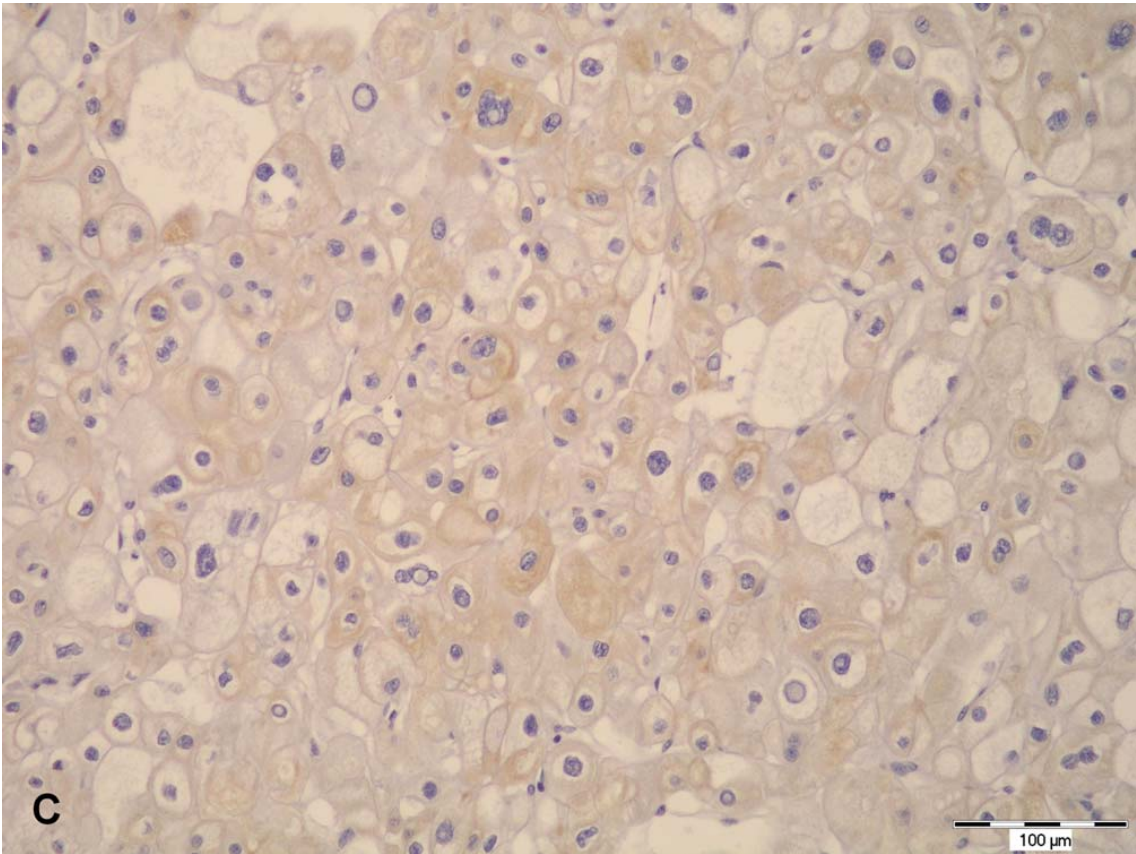
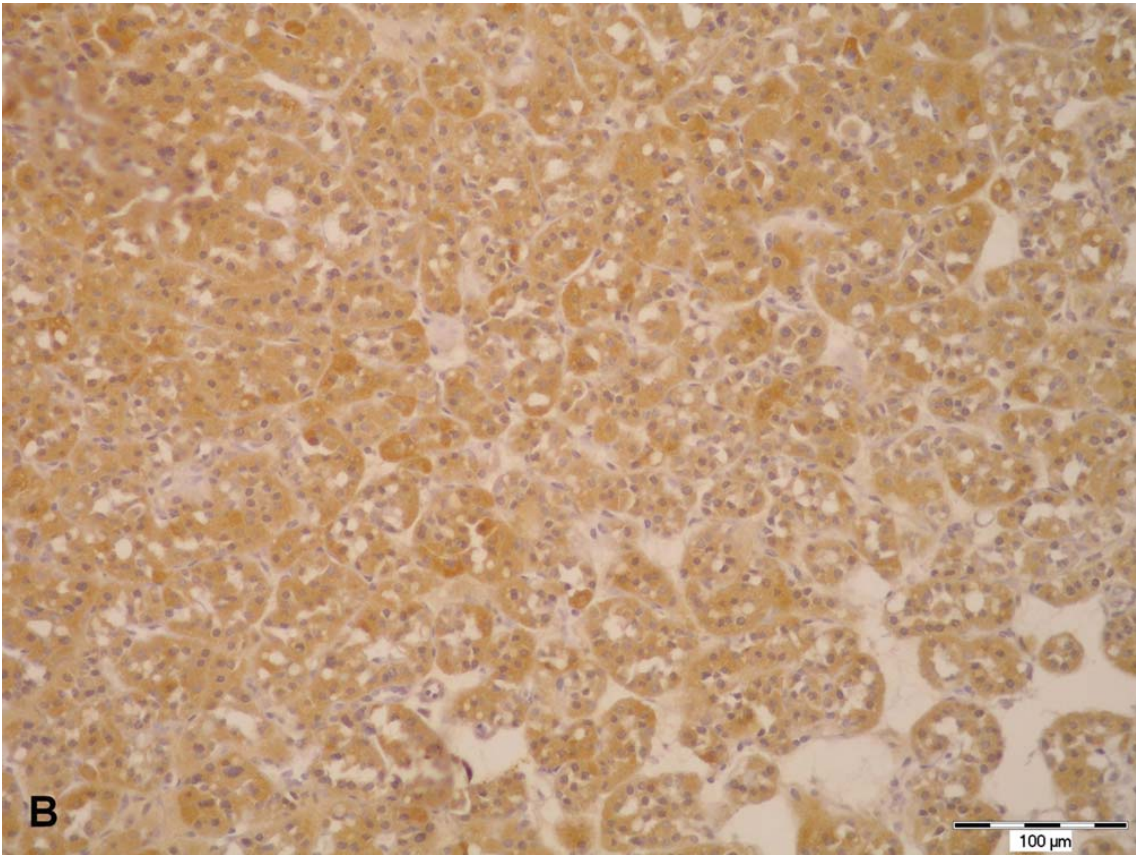
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Fig 1. The majority of renal oncocytomas showed strongly positive reaction for MAGE-A3/4 (A) and NY-ESO1 (B), while only few cases of chromophobe renal cell carcinoma were focally weak positive for MAGE-A3/4 (C) and NY-ESO1 (D). (The bars in all figures indicate 100  $\mu$ m.)







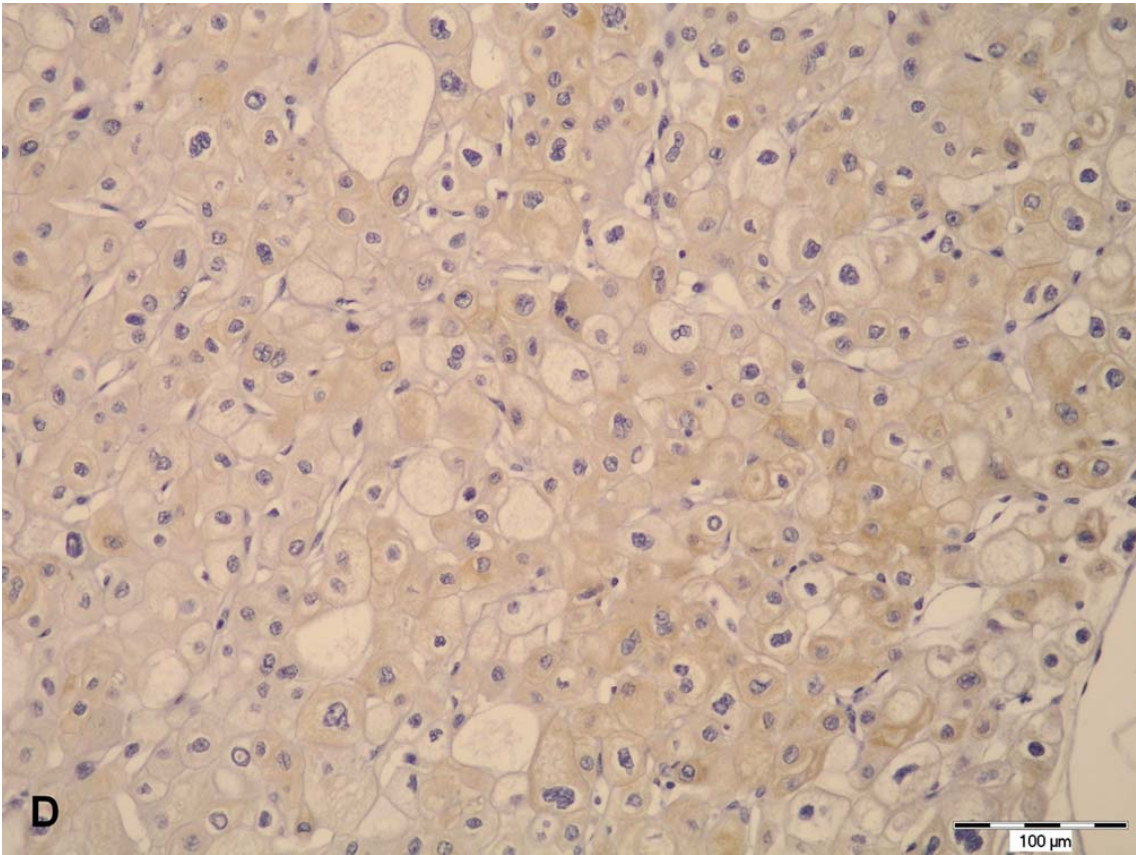


Table 1. Clinicopathologic characteristics and results of immunohistochemical staining for MAGE-A3/4 and NY-ESO-1 in renal oncocytoma

No.	Age	Gender	Tumor Size (cm)	MAGE-A3/4*	NY-ESO-1*
1.	80	F	6.0	+	++
2.	47	M	4.3	-	-
3.	74	F	2.6	++	++
4.	52	M	6.0	++	+++
5.	62	M	3.5	+	+
6.	69	F	2.0	+++	+++
7.	66	M	3.0	-	-
8.	58	F	4.2	+	+
9.	61	F	1.7	+++	+++
10.	62	F	3.0	++	+++
11.	57	M	4.0	+++	+++
12.	77	M	0.9	+++	+++
13.	68	F	2.2	+++	+++
14.	68	M	3.0	++	+++
15.	69	F	6.5	+++	+++
16.	55	F	8.0	++	++
17.	70	F	1.5	+++	+++

\* (-) = no staining in tumor cells

(+) = up to 10% of tumor cells positive

(++) = >10-50% of tumor cells positive

(+++)= more than 50% of tumor cells positive

Table 2. Clinicopathological characteristics and results of immunohistochemical staining for MAGE-A3/4 and NY-ESO-1 in chromophobe renal cell carcinoma

No.	Age	Gender	Tumor Size (cm)	TNM	Nuclear grade <sup>§</sup>	MAGE-A3/4*	NY-ESO-1*
1.	60	F	12	T2N0Mx	2	++	++
2.	62	M	2.7	T1NxMx	2	++	+++
3.	47	F	17	T3N0Mx	4	+	+
4.	47	F	1.7	T1NxMx	3	++	++
5.	44	F	6.8	T1NxMx	2	-	-
6.	63	M	10	T3aN0Mx	2	-	-
7.	74	F	4.5	T1NxMx	2	+	-
8.	60	F	7.0	T1N0Mx	2	+++	+++
9.	55	F	6.5	T1N0Mx	2	+	+
10.	51	F	8.0	T2NxMx	2	-	-
11.	66	M	6.2	T1N0Mx	3	-	-
12.	76	M	4.5	T1NxMx	2	-	-
13.	49	F	7.5	T2NxMx	3	-	-
14.	75	M	11	T2N0Mx	3	-	-
15.	34	F	12	T2N0Mx	3	-	-
16.	52	M	6.0	T1NxMx	2	-	-
17.	56	F	5.5	T1NxMx	2	-	-
18.	76	M	7.2	T3aNxMx	3	-	-

<sup>§</sup> Nuclear grade assessed according to the Fuhrman classification system

\* (-) = no staining in tumor cells

(+) = up to 10% of tumor cells positive

(++) = >10-50% of tumor cells positive

(+++)= more than 50% of tumor cells positive