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Expression of erythropoietin and neuroendocrine markers in clear cell renal cell carcinoma

PATRICIA G. MJØNES,[1,2] IVAR S. NORDRUM,[2,3] GUNNAR QVIGSTAD,[4] ØYSTEIN SØRDAL,[4] LISELOTTE LINNEA MÅRTENSSON RIAN[5] and HELGE L. WALDUM[1,4]

[1]Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology (NTNU), Trondheim; [2]Department of Pathology and Medical Genetics, St. Olavs Hospital, Trondheim University Hospital, Trondheim; [3]Department of Laboratory Medicine, Children's and Woman's Health, NTNU, Trondheim; [4]Department of Gastroenterology and Hepatology, St. Olavs Hospital, Trondheim University Hospital, Trondheim; and [5]Department of Urology, St. Olavs Hospital, Trondheim University Hospital, Trondheim, Norway

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The aim of the study was to investigate the expression of erythropoietin and neuroendocrine markers in clear cell renal cell carcinoma (CCRCC). We retrospectively reviewed the medical records and re-evaluated histopathological specimens of 33 patients with CCRCC and compared with those of 11 cases of non-CCRCC. All patients were treated with a partial or radical nephrectomy at St. Olavs Hospital, Trondheim University Hospital, between 2010 and 2016. Thirty-three patients who were diagnosed with CCRCC had a total of 35 tumours, where 34 of the tumours were CCRCC and one was papillary adenoma. Thirty-three (97%) of 34 CCRCCs were positive for erythropoietin, and the same 33 (97%) tumours demonstrated strong expression for neuron-specific enolase (NSE). Two (6%) of 34 CCRCCs had a positive reaction for synaptophysin, and three (9%) of 34 were positive for CD56. Erythropoietin and NSE were negative in non-CCRCCs, and chromogranin A was negative in all tumours. The above findings suggest that there is a strong association between CCRCC and the expression of erythropoietin and NSE.

Key words: Erythropoietin; chromogranin; neuron-specific enolase; synaptophysin; clear cell renal cell carcinoma.

Patricia G. Mjønes, Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, NTNU, NO-7491 Trondheim, Norway. e-mail: patricia.mjones@ntnu.no

Neuroendocrine (NE) cells and neuroendocrine tumours (NETs) have been described in a variety of organs. In particular, the role of the neuroendocrine (NE) cell in the stomach has been studied extensively for decades. The enterochromaffin-like (ECL) cell and its role in carcinogenesis have been of interest (1). Studies have revealed that an important part of human gastric carcinomas displays NE markers, and more specifically ECL cell markers (2, 3), indicating that these carcinomas originate from ECL cells. Much less, however, is known about the NE cells and NETs in the kidneys (4). Interestingly, a primitive neural crest–derived tumour has been described (5). Likewise, primary NETs in the kidney are seldom, but well-known (6).

Approximately 90% of the body's erythropoietin (EPO) is produced by the kidneys (7). Curiously, the cell type producing EPO has not yet been settled with certainty. The proximal tubular cells are claimed to be the main source of EPO production by some reports (8, 9). Other reports have presented evidence in favour of the glomerular cells (10), mesangial cells (11) or the renal interstitial cells (12–14). The prevailing opinion at present is that interstitial, peritubular cells located to the inner renal cortex and outer medulla produce this hormone (12–14). There is still confusion regarding the EPO-producing cells and their regulation. However, the EPO-producing cell seems to play a role in clear cell renal cell carcinoma (CCRCC) as 5% of patients with such tumours display polycythemia, which may be regarded as a parallel to hormone overproduction syndrome in patients with

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NETs (15). There are also other similarities between NETs and CCRCCs in that both tumour types tend to grow slowly, but nevertheless metastasize at an early stage (16, 17). NETs may also display a morphology which resembles CCRCCs (18, 19). Based on the similarities of CCRCCs to NETs in other organs, and the description of NE markers in CCRCCs (20), we wished to explore this aspect further both in normal kidneys and CCRCCs.

The aim of our study was twofold. First, we wanted to investigate the expression of EPO and general NE markers in patients with CCRCC. Second, we wanted to see if we could find the cell responsible for producing EPO in the normal human kidney tissue.

METHODS

Patients

In this study, there were a total of 44 patients divided into three groups. All patients were treated with either radical nephrectomy or partial renal resection for renal cell carcinoma (RCC) at St. Olavs Hospital, Trondheim University Hospital, between 2010 and 2016. The first group consisted of 24 patients who had been through surgery between 2010 and 2014. All the patients with CCRCC in the given time period were identified by going through our records at the urology and pathology departments, and only patients with a haemoglobin (Hb) value of 15 g/dL or more just prior to surgery were included in this group. A cut-off value for Hb was set at 15 g/dL or more to include enough patients with possible symptoms of polycythaemia due to EPO overproduction. Of 83 patients registered with CCRCC, 24 (29%) patients had Hb value more than 15 g/dL. Information about the Hb value was extracted from the patient's medical records. Their median age was 56.5 (range 37–81) years at the time of diagnosis. The second group consisted of nine patients with CCRCC treated with radical or partial renal resection in 2016. All patients in this group had Hb value less than 15 g/dL and were chosen from a group of 25 patients with renal cell carcinoma (RCC) who had given consent to participate in the study prior to surgery. Their median age was 60.0 (range 45–73) years at the time of diagnosis. The purpose of the second group was to serve as a control to the first group and to see whether the Hb value influenced the expression of EPO. The third group consisted of 11 patients with non-CCRCC treated with either radical nephrectomy or partial renal resection for non-CCRCC at St. Olavs Hospital, Trondheim University Hospital, between 2010 and 2016. The third group of patients was selected from screened non-CCRCCs. The patients in the third group were identified by going through our records at the pathology department, and Hb value of 15 g/dL or more was not used as a cut-off for this group as this would exclude too many patients. Their median age was 71.0 (range 39–82) years at the time of diagnosis. The study was approved by the Regional Committee for Medical and Health Research Ethics in Trondheim, Norway (project number in REK: 2015/211).

Archival formalin-fixed, paraffin-embedded (FFPE) tissue from all 44 patient's kidneys with RCC was retrospectively re-examined, graded according to Fuhrman (21), and staged according to International Union Against Cancer (UICC), *TNM Classification of Malignant Tumours*, seventh edition (22). Tumour size, which was defined as the greatest diameter recorded in the pathological specimen, was noted. The medical records were reviewed, and appropriate immunohistochemical (IHC) staining with antibodies against EPO, chromogranin A (CgA), synaptophysin, neuron-specific enolase (NSE) and CD56 was performed. *In situ* hybridization (ISH) with an EPO probe was also performed. We hoped that by using more sophisticated methods with regard to immunohistochemistry (IHC) (2) and *in situ* hybridization (ISH) (23), some of these tumours would display positivity for general NE markers.

Histopathology and immunohistochemistry

Four-μm thick sections were cut from tissue blocks of paraffin, and the sections were subsequently transferred to SuperFrost Plus slides (Thermo Scientific, Braunschweig, Germany). The slides were allowed to dry overnight at room temperature and then for 60 min at 60 °C. After being deparaffinized in NeoClear (Merck KGaA, Darmstadt, Germany), the sections were rinsed in decreasing grades of alcohol down to water, followed by blocking of endogenous peroxidase by putting the slides in a bath of 0.3% H_2O_2 for 10 min. Epitope retrieval was performed by boiling the sections in Tris/EDTA (pH 9) for all the various antibodies used in this study: erythropoietin, CgA, synaptophysin, NSE and CD56, in a microwave oven at 160W for 15 min. This was followed by cooling the sections for 15 min at room temperature. After washing the sections in a wash buffer solution, they were incubated with a primary antibody at 4 °C overnight (erythropoietin) or for 1 h at room temperature (CgA, Synaptophysin, NSE and CD56). The antibodies used for this study were erythropoietin (Ab20473; Abcam, Cambridge, UK, 1:500) and antibodies against general NE markers: chromogranin A (M0869; Dako, Glostrup, Denmark,

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1:200), Synaptophysin (M7315; Dako, 1:200), NSE (M0873; DAKO, 1:200) and CD56 (M7304; Dako, 1:50). The immunoreactions against the antibodies were further amplified using Mouse Link (K8021; Dako) and afterwards visualized using an EnVision-HRP kit with DAB+ (K5007; Dako). Mouse IgG1 (Ab81216; Abcam) was used as a negative isotype control for EPO. Mouse IgG2b (X0944; Dako) was used as a negative isotype control for CgA, and mouse IgG1 (X0931; Dako) was used as negative isotype control for synaptophysin, NSE and CD56. A TNT wash buffer solution was used to wash the sections between the last two steps. A known carcinoid of the appendix was used as a positive control for the NE markers, and human first trimester placental tissue and a CCRRC with positive staining for EPO was used as a positive control for EPO.

Grading in accordance with the Fuhrman grading system was used when evaluating the tumours (21). The Fuhrman grading system is four tiered, and it assesses nuclear size and shape and nucleolar prominence. It assumes that there is a correlation between the different grades and each parameter examined (24). A median of 3 (range 1–8) slides were examined before the tumours were graded. As recommended by Delahunt et al., the worst grade observed in the sections was recorded, irrespective of area of tumour that was assessed (25). The staining was classified as positive or negative. When appropriate, the positive staining was further classified as weak (2–10% of tumour cells staining positive), moderate (10–50% of tumour cells staining positive) or strong (more than 50% of tumour cells staining positive). If less than 2% of tumour cells stained positive, the staining was classified as negative. The staining pattern for EPO was further validated by using *in situ* hybridization (ISH) with an EPO probe.

In situ hybridization

In situ hybridization (ISH) was only performed on the patients of the first study group. For ISH, RNAscope 2.0 HD Assay-Brown (ACDbio, Newark, California, USA) was used, and the method was performed according to the protocol provided by ACDbio with a few minor modifications. As for IHC, 4-μm tissue sections were cut from tissue blocks of paraffin, and the sections were subsequently transferred to SuperFrost Plus slides. The slides were allowed to dry overnight at room temperature (at approximately 25 °C). After baking the sections at 60 °C for 60 min, the slides were deparaffinized in NeoClear (2 × 10 min) before dehydrated in 100% EtOH (absolute alcohol) for 2 × 2 min. Afterwards, the sections were air-dried for 5 min, followed by incubation with Pretreat 1 for 10 min at room temperature. Thereafter, the slides were immersed in boiling Pretreat 2 (for RNA retrieval) for 30 min. After cooling the sections down in distilled water (30 s), they were incubated with Pretreat 3 (for protein digestion) for 30 min at 40 °C. This was followed by rinsing the slides in distilled water.

The tissue sections were incubated with the target probe against human erythropoietin (Probe-Hs-Epo; ACDbio, catalogue number: 414201), negative control probe (negative control probe-DapB; ACDbio, catalogue number: 310043) and positive control probe (positive control probe–Hs-PPIB; ACDbio, catalogue number: 313901) for 2 h at 40 °C, and then rinsed twice in a wash buffer (from ACDbio, following the kit). Signal amplification was done according to the recommendations from ACDbio. The tissue sections were incubated with Amp 1 (preamplifier) for 30 min at 40 °C, Amp 2 (background reducer) for 15 min at 40 °C, Amp 3 (amplifier) at 40 °C, Amp 4 (label probe) for 15 min at 40 °C, Amp 5 for 30 min at room temperature, and finally, Amp 6 for 15 min at room temperature. After each of the steps, the sections were rinsed in wash buffer. For signal detection, a DAB mixture (following the kit) was used, and counterstaining was done by staining the sections with haematoxylin.

The positive and negative control probes were used to detect RNA quality and background signals. Positivity was defined by the presence of dot-like/punctate cytoplasmic and/or nuclear staining that was above that of the dapB slide (negative control).

Statistical analysis

IBM SPSS statistics version 22 (Chicago, IL, USA) was used for calculation of mean and median values of the different parameters, as well as range. Spearman's rank nonparametric test was performed to look for association between the various variables.

RESULTS

Patient characteristics

Of the 24 patients included in the first study group, five (21%) of the patients were women, the remaining 19 (79%) were men. A total of 26 tumours were resected from these patients. Two of the patients had two kidney tumours and the remaining 22 patients had one kidney tumour. One patient had both CCRCC and papillary adenoma, and one patient had two CCRCCs. The median age at the

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time of surgery was 56.5 (range 37–81) years, and the mean Hb value was 15.6 g/dL (SD: 0.4 g/dL). Nine (35%) tumours were operated by radical nephrectomy and 17 (65%) by partial nephrectomy. During follow-up, two (8%) of the patients had died from the disease: one from metastatic disease and one due to complications from surgery.

A total of nine patients diagnosed with CCRCC were included in the second study group. All patients in this group had Hb value less than 15 g/dL, with a mean Hb value of 12.6 (SD: 2.2) g/dL. Seven (78%) patients were men and the remaining two (22%) were women. A total of nine tumours were resected from these patients by radical (44%) or partial (56%) nephrectomy, and the median age at time of surgery was 60.0 (range 45–73). During follow–up, all were alive and well.

In the third study group, one (9%) patient was woman, the remaining ten (91%) were men. A total of 11 tumours, which all were non-CCRCC, were resected from these patients either by radical (18%) or partial (82%) nephrectomy. The median age at the time of surgery was 71 (range 39–82) years, and the mean Hb value was 14.1 (SD: 1.1) g/dL. During follow-up, three (27%) patients with non-CCRCC had died from the disease.

Tumour characteristic

The mean tumour size, which was defined as the greatest diameter recorded in the pathological specimen, was 3.0 (range 1.2–7.5) cm with an SD of 1.6 cm in the first group, 4.9 (range 2.0–7.6) cm with an SD of 2.1 cm in the second group, and 4.4 (range 1.7–10.0) cm with an SD of 3.2 cm in the third group. In the first group, two (8%) of the tumours were Fuhrman grade 1, 19 (76%) tumours were grade 2 and the remaining four (16%) were grade 3. The second group consisted of one (11%) Fuhrman grade 1 tumour, three (33%) grade 2, three (33%) grade 3 and two (22%) grade 4. In the third group, one tumour (9%) was Fuhrman grade 1, six (55%) were grade 2, one (9%) was grade 3 and one (9%) was grade 4. Two tumours diagnosed as chromophobe renal cell carcinoma (ChRCC) were in accordance with current guidelines not graded according to Fuhrman (26). All the patients operated on in the first group were in the pathology report diagnosed as having CCRCC. In one of the patients, a small 3.3-mm papillary adenoma was found as an incidental finding upon histopathological examination of the specimen. All tumours in the second group were CCRCCs. In the third group, seven (64%) of the tumours were papillary renal cell carcinoma (PRCC) type 1, two (18%) were PRCC type 2, and the remaining two (18%) were ChRCC. According to the TNM classification, 20 (80%) tumours in the first group were in the T1a category, three (12%) tumours were in the T1b category, one (4%) tumour in the T2a category, and one (4%) tumour was in the T3a category. The second group consisted of five (56%) T1b tumours, one (11%) T1b tumour and three (33%) T2a tumours. In the third group, eight (73%) of the tumours were in category T1a, one (9%) in T2a, one (9%) in T2b and one (9%) in T3a.

Immunohistological findings

All 46 tumours in all three groups were stained with antibodies against EPO, and for the general NE markers CgA, synaptophysin, NSE and CD56. As the Hb value did not seem to significantly influence the staining results, the results of groups 1 and 2 are described together. Of the 34 CCRCCs from groups 1 and 2 stained with EPO, 33 (97%) of them were positive (Fig. 1A,B). In 28 (82%) of these tumours, more than 50% of the tumour cells stained positive, whereas 10–50% of the tumour cells stained positive in five (15%) of the tumours. One (3%) tumour had a few scattered EPO expressing cells in the tumour tissue, but this amounted to less than 2% of the total tumour tissue. The papillary adenoma from the first group and PRCCs and ChRCCs from the third group did not express EPO. Positivity for NSE was detected in 33 (97%) of the CCRCCs investigated (Fig. 1C), but was negative in all the non-CCRCCs. Expression of NSE was found in the same tumours positive for EPO. Focal immunopositivity for synaptophysin was detected in two (6%) of the CCRCCs, both tumours from the first group (Fig. 2). The tumours positive for synaptophysin were also positive for NSE. CD56 was expressed in three (9%) CCRCCs, all tumours were from the second group of patients (Fig. 3). None (0%) of the tumours in any of the groups expressed CgA. A summary of staining results is given in Table 1.

When looking at the association between the different variables in all the samples together, there was a significant positive correlation between the expression of EPO and NSE ($r = 0.84$, $p < 0.001$). There was also a weak positive correlation between the expression of EPO and Hb value ($r = 0.35$, $p = 0.02$) and NSE and Hb value ($r = 0.38$, $p = 0.01$). There was no significant correlation between the other variables.

In addition to the above findings, a few scattered peritubular interstitial cells with positive expression for EPO were observed (Fig. 4). These cells were mainly located in the inner renal cortex.

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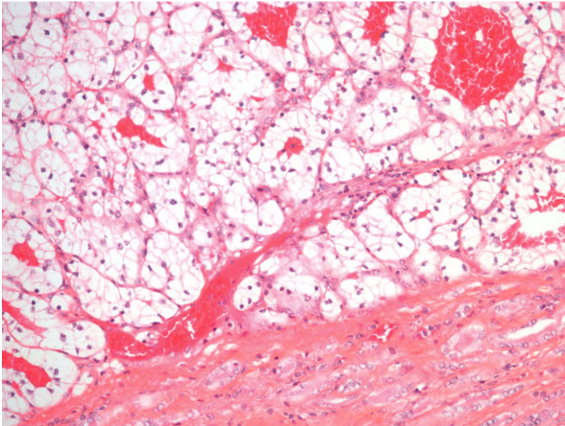
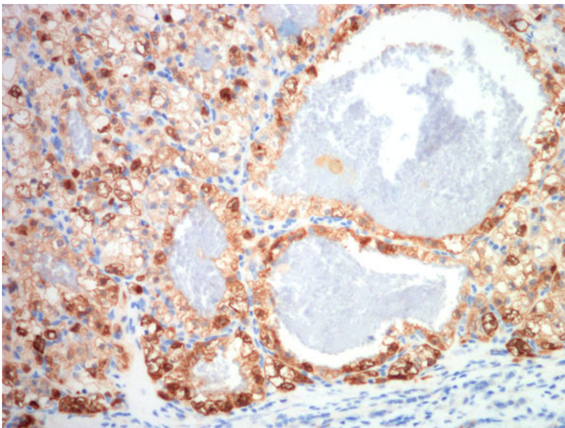
A



B



C

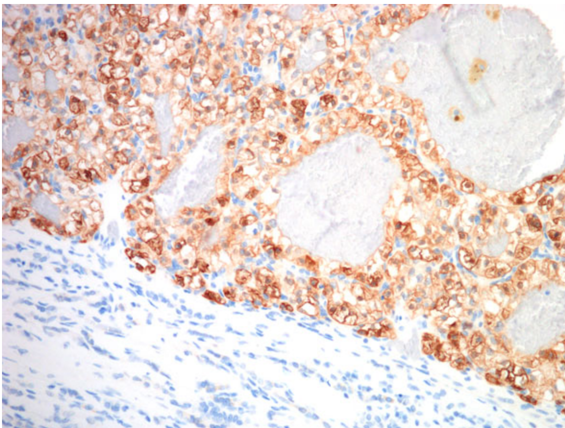


Fig. 1. Clear cell renal cell carcinoma examined by haematoxylin and immunohistochemistry. (A) Hematoxylin and eosin, ×20. (B) Erythropoietin, ×20. (C) Neuron-specific enolase, ×20.

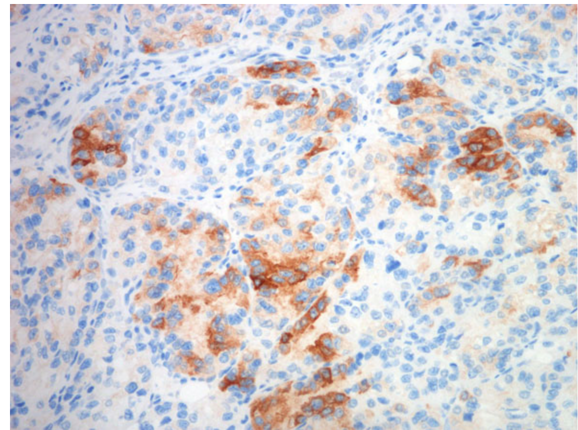


Fig. 2. Synaptophysin expression in CCRCC, ×20.

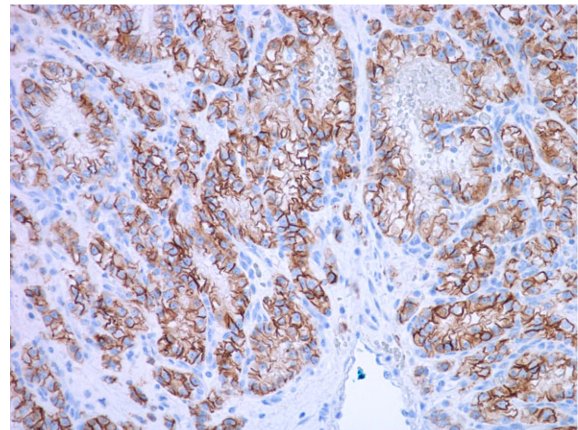


Fig. 3. CD56 expression in CCRCC, ×20.

In situ hybridization findings

Of the 25 CCRCCs in group 1, only seven (28%) tumours demonstrated a weak EPO expression (Fig. 5). In all the tumours, the positive control probe was expressed in low quantities both in tumour and normal kidney tissue, indicating a rather poor quality of the mRNA.

DISCUSSION

Our study demonstrates that EPO and NSE are expressed in the majority of cases with CCRCC. Even the one tumour which was considered negative with regard to EPO and NSE had a few scattered EPO and NSE expressing cells in the tumour tissue, and thus, all the CCRCCs did express EPO and NSE to some degree. All the cases with papillary or chromophobe type morphology were negative for EPO and NSE. The finding of EPO expression in CCRCC is supported by previous studies (27, 28). In one of the studies (27), however, only 33% of the tumours examined expressed EPO.

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Table 1. Immunohistochemical expression of erythropoietin and neuroendocrine markers in clear cell and non-clear cell renal cell carcinoma

Immunohistochemistry/ *in situ* hybridization	Positive	Negative
Immunohistochemistry		
Group 1		
Erythropoietin	24 (96%)	1 (4%)[1]
Chromogranin A	0 (0%)	25 (100%)
Synaptophysin	2 (8%)	23 (92%)
Neuron-specific enolase	24 (96%)	1 (4%)[1]
CD56	0 (0%)	25 (100%)
Group 2		
Erythropoietin	9 (100%)	0 (0%)
Chromogranin A	0 (0%)	9 (100%)
Synaptophysin	0 (0%)	9 (100%)
Neuron-specific enolase	9 (100%)	0 (0%)
CD56	3 (33%)	6 (67%)
Group 3		
Erythropoietin	0 (0%)	11 (100%)
Chromogranin A	0 (0%)	11 (100%)
Synaptophysin	0 (0%)	11 (100%)
Neuron-specific enolase	0 (0%)	11 (100%)
CD56	0 (0%)	11 (100%)
***In situ* hybridization**		
Group 1		
Erythropoietin	7 (28%)	18 (72%)

[1]Even the cases considered negative for erythropoietin and neuron-specific enolase expressed these proteins in a few tumour cells, but this amounted to less than 2%.

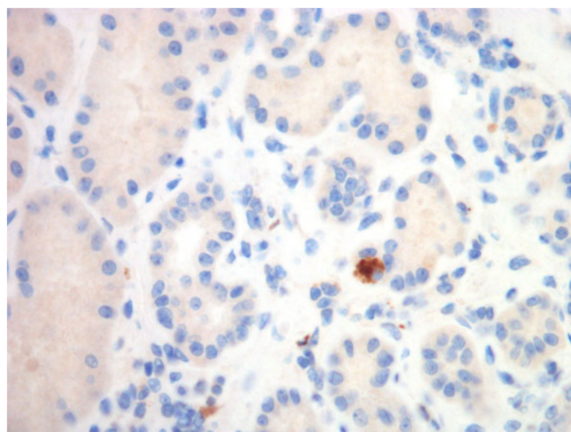

Fig. 4. Interstitial cells with positive EPO expression, ×40.

In the other study (28), 86% of CCRCC displayed positivity for EPO. Papworth et al. also found that a significantly higher proportion of CCRCC had a strong EPO expression compared to RCC of the papillary type. It is unclear if all the tumours in the first mentioned study (27) were CCRCC as this is not clearly specified in their paper. The discrepancy between the results may be due to methodological issues with regard to primary and/or secondary antibodies. The above studies do not specify the antibodies utilized. We have also used a mouse link in our study in order to enhance the signal of EPO, thus rendering more tumour cells positive. The finding of EPO in CCRCC makes sense, as this cancer type is associated with mutation in the Von Hippel–Lindau (VHL) gene. A mutation in this gene will in turn lead to accumulation and stabilization of hypoxia inducible factors (HIF), thus mimicking a hypoxic state in the cell. As a result, EPO, vascular endothelial growth factor (VEGF) and a number of other hypoxia inducible genes are transactivated (29). Almost all the CCRCCs in our study demonstrated positive expression for EPO when IHC was performed. When ISH was done on the same tumours, only seven (28%) of them were positive. This discrepancy could be due to the poor mRNA quality of the FFPE kidney tissue due to variable fixation time and variable condition and age of the tissue blocks. After numerous of experiments performed on human kidney tissue, and similar size tissue from the intestines and placenta, the mRNA quality of the kidney tissue was poor in comparison to tissue samples from other locations.

All but one of the CCRCCs showed convincing and strong positive expression for NSE. Even the one tumour considered to be negative expressed NSE in a few scattered cells. Enolases are glycolytic enzymes that are widely distributed in mammalian tissue. They are dimers consisting of three distinct subunits: α, β and ϒ. NSE is the ϒ unit of enolase and is present at high levels in neuronal cells as αϒ or ϒϒ forms. The antibodies against NSE used for IHC are against the ϒϒ form, which is the subunit specific for NE cells and neurons. High levels of this subunit are also found in tumours derived from these cells (30). The specificity of this antibody, however, is reduced due to cross-reactivity with the αϒ form of enolase, which is found in smooth muscle cells, myoepithelial cells and lymphocytes (31). Moderate levels of NSE may also be found in tumours that are not derived from neuronal or NE cells. Haimoto et al. (30) were able to demonstrate NSE (ϒ-enolase) in the epithelial cells of loops of Henle and macula densa cells. They did not, however, find NSE in epithelial cells of proximal tubules, where most believe CCRCCs are derived from (32). As seen in our study, and in studies by Ronkainen et al. (20) and Haimoto et al. (30), a high number of CCRCCs express NSE. This could indicate that NSE expression occurs during renal oncogenesis or that CCRCC originates from a different cell than the proximal tubular cell.

NSE is often regarded as a rather unspecific marker for NE differentiation. NSE is, however,

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observed in the majority of NE tumours (33), and because it is a cytosolic marker, it can stain even degranulated tumour cells (34). In a study by Abbona et al., 21/40 (53%) non–small-cell lung carcinomas expressed NSE, 4/40 (10%) expressed synaptophysin and 5/40 (13%) expressed CgA (35). All the tumours expressing synaptophysin were also positive for NSE. The finding of NE markers in non–small-cell lung carcinomas is supported by another study done at our department (36). None of the tumours in our study expressed CgA, and only two tumours expressed synaptophysin in approximately 10% of the tumour cells. This result is in agreement with the study done by Ronkainen et al. (20).

As observed in previous studies, CD56 is expressed in a percentage of clear cell renal cell carcinomas (20). CD56, also known as NCAM, is a neural cell adhesion molecule which is thought to be a rather sensitive marker of neuroendocrine differentiation, especially in small-cell lung carcinomas (37). Its specificity has, however, been questioned as this marker is expressed in a number of other tumours including CCRCC. In our study, only three (9%) cases of CCRCC expressed CD56, which is lower than the study done by Ronkainen et al. (20).

In our study population, there were almost five times as many men as women, which is to be expected as there are almost twice as many men as women that are affected by this disease in the first place (38). Our Hb cut-off of 15 g/dL will also favour more men being included in the study compared with women, as the normal Hb range is in general higher in men compared with women (men normal range: 13.4–17.0 g/dL, women normal range: 11.7–15.3 g/dL) (39). By choosing a lower Hb value for the female population in our study group, more women would have been included in the study.

The clear cell morphology seen in CCRCC has also been observed in some NETs, mostly occurring in patients with VHL disease (18, 30). As much as 60% of the NETs associated with VHL disease display a spectrum of clear cell morphology (40). The clear cell morphology is thought to be due to an accumulation of lipid and glycogen, which is removed from the cells during processing, subsequently giving the cytoplasm an empty/clear appearance (41). This clear cell morphology seems to be an important feature of both CCRCCs and tumours associated with VHL disease (18, 42). In a study by Tun et al. using a genome-wide biological pathway analysis package, they discovered biological alterations that may be associated with loss of epithelial differentiation in early-stage CCRCC. They also found a molecular signature consistent with adipogenic transdifferentiation, which in turn may explain the cytoplasmic accumulation of lipid seen in these tumours (42). VHL disease is associated with multiple highly vascularized tumours throughout the body (29), and a mutation in a tumour suppressor gene which is located on chromosome 3p25 (43). Of patients with sporadic CCRCC, the VHL gene is mutated in 50–80% of cases (44) and hypermethylated in 19% of cases (45). A dysfunctional VHL gene may lead to accumulation and stabilization of hypoxia inducible factors (HIF), which in turn accumulates in the nucleus, and subsequently binds to hypoxia response elements (HRE) of the DNA and recruits p300 (CBP) to the C-terminal transactivation domain (CTAD). As a result, more than 60 hypoxia inducible genes are transactivated (29, 46).

There is controversy as to the cellular origin of VHL-associated CCRCC. Based on tumour morphology, the common opinion has been that CCRCC arise from proximal renal tubular cells. Renal cysts and VHL-associated CCRCC have, however, been found to express molecular markers found in both proximal and distal tubular cells (47, 48). Rankin et al. (49) found in mice with a conditional inactivation of the VHL protein (PEPCK-Vhlh mutant mice) data supporting the prevailing opinion that the fibroblast-like interstitial cells located to the inner renal cortex/outer medulla are the cells responsible of producing EPO in the adult kidney. CCRCCs, which by most are considered to be derived from renal epithelial cells, may require additional genetic alterations/changes in order to express EPO (14, 49–51). Furthermore, their study demonstrates that these mice develop tubular micro-cysts that are deficient of the VHL protein. These cysts also show evidence of dedifferentiation and increased proliferation, as well as expressing HIF target genes and protein markers seen in multiple segments of the nephron.

In a few of the normal tissue sections examined, a few scattered interstitial cells with positive expression for EPO were observed (Fig. 4). Whether this staining represents true EPO production or non-specific staining is uncertain. Identification of exactly which cells that actively produce EPO is an unsolved issue. Many previous attempts have been made to find the EPO-producing cell in the kidney. The difficulty in finding the EPO-producing cell is likely due to a low sensitivity in the detection of the hormone. In the normoxic state, only low levels of EPO is found in the serum and urine, suggesting that only low levels of EPO is needed for production of red blood cells. In addition, studies indicate that the number of EPO-producing cells rather than the

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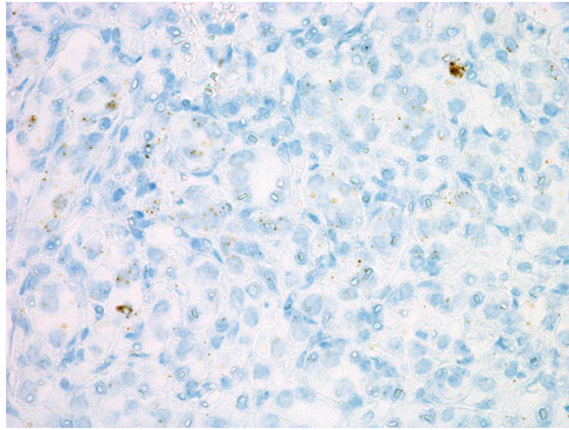

Fig. 5. Clear cell renal cell carcinoma examined by *in situ* hybridization, erythropoietin, ×40.

expression levels of EPO is important when more EPO is needed in the body (52, 53). In a study done by Obara et al. (53), they identified the renal interstitial cells as the cells producing EPO. They also found that a single nucleotide mutation in the promoter GATA box can cause ectopic expression of green fluorescent protein under the control of an EPO gene locus in the distal renal tubules, collecting ducts, and epithelial cells of other tissue types. CCRCC is by many thought to be derived from proximal tubular cells. Some of these tumours are discovered due to polycythaemia secondary to EPO production. If the tubular cells themselves are not the cells in charge of producing EPO, the EPO production in these cells could be due to (as suggested by Obara et al.) some defects in the GATA signalling pathway in this epithelial tumour, leading to ectopic EPO production.

The classification of RCC is under constant change, and new subtypes are emerging (25). Out of all the different subtypes, renal cell carcinoma of the clear cell type is the most common accounting for approximately 75% of all cases (54). Improvement in the understanding of tumour morphology, immunohistochemistry (IHC) and molecular pathology have caused an increase in the number of tumour entities that are currently recognized. Unfortunately, improvement in the treatment of cancers in general is marginal, at least for the more prevalent cancers. We believe that major progress depends on thorough knowledge of the cell of origin. The paradigm till now is that malignant tumours develop from stem cells that stop in differentiation (55). Research on NE cells in the gut has, however, demonstrated that normal NE cells have the ability to proliferate through stages of hyperplasia and dysplasia, and from rather benign NETs to highly malignant neuroendocrine carcinomas (NECs). This in turn challenges the concept that all tumours originate from stem cells.

CONCLUSIONS

In summary, we found that almost all the tumours examined with morphology consistent with CCRCC demonstrated strong expression for EPO and NSE, while PRCCs and ChRCCs were negative for the same markers. These findings suggest that there is a strong connection between CCRCC and expression of EPO and NSE in these tumours. In the cases where the RNA quality was good enough, the same tumour tissue also expressed EPO mRNA. Although a very few scattered EPO expressing cells were observed in the renal interstitium, we cannot be sure as to which cells produce EPO in the normoxic state in man.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

PM evaluated the haematoxylin- and eosin-stained sections, as well as performing and evaluating the immunohistochemical staining and *in situ* hybridization. Statistical analysis was done by PM. IN and HW designed the study. LR and IN were involved in patient selection for the study, and GQ and ØS were involved in evaluating the quality of the immunohistochemical and *in situ* hybridization techniques. All authors revised and approved the final manuscript.

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