Immunohistochemical Evaluation of EGFR and HER-2/NEU Expression in Renal Cell Carcinomas and Their Prognostic Significance

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Abstract

Renal cell carcinoma (RCC) represents about 1% to 3% of all visceral cancers and accounts for 85% of renal cancers in adults. Literatures related to incidence and epidemiology of renal cell carcinoma have confirmed its increasing incidence. The purpose of this study is to evaluate expression of EGFR and HER-2/NEU in cases of renal cell carcinoma and to correlate their expression with pathological prognostic factors. This study consisted of 40 cases of renal cell carcinoma. In the current study 18 cases showed immunoreactivity for EGFR (45% of total cases) while 25 cases showed immunoreactivity for HER-2/NEU (63% of total cases). Statistical analysis for a possible correlation between EGFR and HER-2/NEU expression and the various prognostic factors in this study (tumor stage, nuclear grade, macrocapsular invasion, microcapsular invasion, macrovascular invasion and microvascular invasion) had revealed non significant correlation.

Key Words: Renal – EGFR – HER2/NEU – Carcinoma.

Introduction

RENAL cell carcinoma (RCC) represents about 1% to 3% of all visceral cancers and accounts for 85% of renal cancers in adults. There are 30,000 new cases and 12,000 deaths per year from the disease [1]. Literatures related to incidence and epidemiology of renal cell carcinoma (published between May 1, 2003 and April 30, 2004) have confirmed the increasing incidence of renal cell carcinoma [2]. Therefore, several studies have focused on evaluating indicators of biologic aggressiveness of renal cell carcinoma. A variety of proteins and carbohydrates have been investigated for their use as prognostic tumor markers [3].

Patients with clear cell renal cell carcinoma tend to have a worse prognosis than patients with other histologic subtypes of renal cell carcinoma, with 5-year disease-specific survival rates of 50-69%, compared with 67-87% for papillary renal cell carcinoma and 78-87% for chromophobe renal cell carcinoma [4].

Epidermal growth factor receptor (EGFR) is a protein tyrosine kinase that plays a crucial role in signal transduction pathways that regulate key cellular functions such as survival and proliferation [5].

EGFR is a 170-kDa cell surface glycoprotein containing three well identified parts: An extracellular legand-binding domain, a hydropic membrane-spacing domain and a cytoplasmic protein containing the tyrosine kinase activity. The effect of EGFR activation on tumoral cells are multiple and convergent, thus favoring uncontrolled cell growth with an increase in cell mobility and cell proliferation, a decrease in apoptotic machinery and stimulation of angiogenesis [6].

EGFR expression is reported in up to 95% of renal cell carcinoma and is considered high in up to 60% [7]. Among recent advances in the molecular targeted therapy of cancer, applications centered on EGFR are currently the most promising and the most advanced at the clinical level [5].

HER-2/NEU oncogene encodes a transmembrane glycoprotein with tyrosine kinase activity and extended homology in structure and sequences to the EGFR [8]. Cancers that show HER-2 amplification generally have a poor prognosis and shorter overall survival. HER-2/NEU gene amplification and protein overexpression have been associated constantly with high tumor grade, DNA aneuploidy, high cell proliferation rate, negative assays for nuclear protein receptors for estrogen and progesterone, p53 mutation, topoisomersae Ila amplification and alterations in a variety of other molecular biomarkers of barest cancer invasiveness and metastasis [9].
HER-2 amplification is also a predictive marker of responsiveness to selected forms of therapy [10].

The purpose of this study is to evaluate expression of EGFR and HER-2/NEU in cases with renal cell carcinoma and to correlate their expression with pathological prognostic factors: The tumor stage (pT), nuclear grade, macrovascular invasion, macrocapsular invasion, microvascular invasion, microcapsular invasion and finally to estimate the potential prognostic relevance of HER-2/NEU and EGFR expression.

Material and Methods

The material of this study consisted of the paraffin blocks of 40 cases that had underwent radical nephrectomy and were diagnosed as renal cell carcinoma. All nephrectomy specimens were formalin fixed, routinely processed and embedded in paraffin. Cases were obtained from the department of Pathology, Faculty of Medicine, Cairo University, in the period from January 2007 to December 2008.

Age, sex, tumor size, tumor stage (pT), gross capsular and gross vascular invasion were registered according to data present in the clinical file and pathology report of each case.

Serial sections of 4 microns thick were prepared from each block, one of them was mounted on glass slide and stained by Hematoxylin and Eosin (H&E) for histological evaluation and another two were mounted on charged glass slides for immunohistochemical staining.

By examining the H&E slides, renal cell carcinomas were graded by Fuhrman nuclear grading system [11] as follows:

Grade 1: Nuclei are small (<10 g.m) and round, with dense chromatin and inconspicuous nucleoli.

Grade 2: Nuclei are slightly larger (15 µm) with finely granular chromatin and small nucleoli.

Grade 3: The nuclei are 20 µm in size and may be oval in shape, with coarsely granular chromatin and prominent nucleoli.

Grade 4: The nuclei are pleomorphic with open chromatin and single or multiple macronucleoli.

Evidence of microcapsular invasion was thoroughly looked for by examining the H&E slides. Finally, the presence of microvascular invasion was evaluated and defined as positive when there were neoplastic cells in an endothelium-lined space and/or in the intra-tumoral microcirculation [12].

**Immunohistochemical staining for EGFR:**

- The sections were deparaffinized in xylene, then were hydrated through a series of graded alcohols (95%-70%), distilled water and phosphate buffered saline (at ph 7.5).
- The slides were then immersed in 10 mM citrate buffer (ph 6) and were twice pretreated by microwaving (oven 800w) for 4 then 8 minutes.
- Between each period of heating, evaporated fluid was replenished.
- After a 25 minute cooling period, the endogenous peroxidase activity was inhibited by incubation in 3% hydrogen peroxide (H₂O₂) for 5 minutes.
- After washing with Tris-buffered saline, the sections were incubated with the primary antibody for 1 hour at room temperature. The primary antibody was a mouse monoclonal antihuman EGFR antibody, supplied in a liquid form (Dako, Denmark) at dilution 1:200.
- The sections were washed in Tris-buffer and incubated with avidin-biotin-peroxidase system (DAKO) for 30 minutes. Peroxidase reaction was detected by addition of diamonobenzidine tetrahydrochloride.
- All slides were rinsed well in tap water for 5 minutes then slightly counterstained with Mayer's Hematoxylin for 1-2 minutes and dehydrated in ascending alcohol.
- The slides were cleared in xylene for 3 changes, then Canada palsam and cover slips were applied.

**Evaluation of expression of EGFR:**

Tumor tissue sections were examined and scored under the microscope (Olympus 21X) at high power magnification, for the presence of membrane immunostaining (that may be associated with cytoplasmic and nuclear staining). The membranous staining of EGFR was classified as 0 for negative, 1 for moderate intensity of the cell membrane immunoreactivity and 2 for strong cell membrane immunoreactivity [13]. The positive control for EGFR staining was a section of human placenta, since strong expression of EGFR has been demonstrated in the syncytiotrophoblasts of the placenta.

**Immunohistochemical staining for HER-2/NEU:**

The same steps were followed, however after washing with Tris-buffered saline, the sections were incubated with the primary antibody for 1 hour at room temperature. The primary antibody was a mouse monoclonal antiserum to HER-2/NEU (DAKO, Denmark) at a dilution of 1:100.

**Evaluation of expression of HER-2/NEU:**

Immunohistochemical staining was scored according to both intensity and the number of cells
stained. No staining or membrane staining in _10% was scored as 0, weak staining of the mem-
brane in >10% of cells was scored as 1+ ; moderate
membrane staining in >10% of cells was scored
as 2+, and strong membrane staining in >10% of
cells as 3+. Cytoplasmic staining was excluded. A
score of 2+ or 3+ was regarded as 'positive' and a
score of 0 or 1+ as 'negative' [14]. The positive
control for HER-2/NEU was a section of breast
tumor tissue expressing this protein.

**Statistical analysis:**

All data were collected, recorded and analyzed
using GraphPad InStat software version (2009).

The significance of the results was assessed by
determining the probability factor, p-value, using
the chi-square test. Also in some situations another
statistical test, the Fisher’s exact test was used.

The results were considered significant with p-
value 0.05 or less and non significant with p-value
>0.05.

**Results**

The present study included 40 cases of renal
cell carcinoma that were referred to Kasr El-Aini
hospital and underwent radical nephrectomy.

The ages of the patients ranged between 29 and
80 years with a mean age of 53.5 years.

The number of male patients was 17 (42% of
cases), while the number of female patients was 23
(58% of cases), with a male to female ratio 1: 1.3.

The tumor sizes ranged from 3.5cm up to 18
cm in maximal diameter, with mean tumor size of
7.5cm. Six cases (15%) were less than 4cm in
maximal diameter, 9 cases (22%) were between 4-
7cm in maximal diameter and 25 cases (63%) were
more than 7cm in maximal diameter.

Review of the pathology reports and examina-
tion of the H&E slides of all cases showed that 24
cases were renal cell carcinomas of the conventional
(clear cell) subtype, 4 cases were papillary renal
cell carcinomas, 3 cases were chromophobe renal
cell carcinomas, 3 cases were sarcomatoid renal
cell carcinomas, 2 cases were multilocular cystic
renal cell carcinomas, 2 cases were collecting duct
carcinomas, and 2 cases were renal cell carcinomas
unclassified.

Fourteen out of 40 cases showed evident gross
capsular invasion (this included 6 cases of the clear
cell subtype, all the sarcomatoid, collecting duct
and unclassified subtypes, in addition to one case
of papillary renal cell carcinoma) and also showed
that only 5 out of 40 cases showed evident gross
invasion of the renal vein at the hilum (all were of
clear cell subtype).

As regards tumor stage (pT), 14 cases were of
stage pT1, 9 cases were of stage pT2, 16 cases
were of stage pT3, and one case was of stage pT4.
Advanced tumor stage, pT3 and pT4, was associated
mainly with clear cell renal cell carcinoma subtype
in addition to 2 cases of the sarcomatoid subtype,
one case of either of the collecting duct and the
unclassified subtypes.

According to Fuhrman, the nuclear grading
system showed that: 4 cases were of nuclear grade
I, 21 cases were of nuclear grade II, 10 cases were
of nuclear grade III and 5 cases were of nuclear
grade IV. It was noticed that low nuclear grade (I
& II) was seen mainly in the conventional, papillary,
chromophobe and multilocular cystic subtypes,
while high nuclear grade was seen in association
with all collecting duct and sarcomatoid varieties,
in the other varieties high nuclear grade was asso-
ciated with the appearance of additional histological
features as high grade sarcomatoid component and
rhabdoid features.

Microscopic evaluation of microcapsular inva-
sion showed that 17 cases showed this feature. The
majority of cases were of the clear cell subtype in
addition to all of the sarcomatoid, collecting duct
subtypes and one case of either of the papillary
and the unclassified subtypes.

Thorough evaluation for evidence of microvas-
cular invasion (MVI), within the vicinity of the
tumor and outside the tumor confines, showed that
11 cases were associated with this feature. The
majority were of the clear cell subtype in addition
to one case of each of the collecting duct, papillary,
sarcomatoid and unclassified subtypes.

**Evaluation of the results of immunostaining for
EGFR:**

<table>
<thead>
<tr>
<th>Score of EGFR immunoreactivity</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score 0 (negative)</td>
<td>22 (55%)</td>
</tr>
<tr>
<td>Score 1 (moderately positive)</td>
<td>12 (30%)</td>
</tr>
<tr>
<td>Score 2 (strongly positive)</td>
<td>6 (15%)</td>
</tr>
<tr>
<td>Total number</td>
<td>40 (100%)</td>
</tr>
</tbody>
</table>

The positive cases included 9 cases of the clear
cell subtype (Figs. 1,2) 2 cases of each of the
collecting duct, chromophobe, sarcomatoid and
unclassified subtypes and one case of multilocular
cystic subtype.
Table (2): Correlation between the expression of EGFR and the different pathological prognostic factors.

<table>
<thead>
<tr>
<th>Prognostic Factors</th>
<th>EGFR expression</th>
<th>Cases negative for EGFR expression</th>
<th>Cases positive for EGFR expression</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>pT1</td>
<td>7</td>
<td>pT2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>pT3</td>
<td>10</td>
<td>pT4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Nuclear grade</td>
<td></td>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>GI</td>
<td>2</td>
<td>GII</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Macrocapsular invasion</td>
<td></td>
<td>Cases positive for macrocapsular invasion</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Microcapsular invasion</td>
<td></td>
<td>Cases negative for macrocapsular invasion</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Macrovascular invasion</td>
<td></td>
<td>Cases positive for macrocapsular invasion</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Microvascular invasion</td>
<td></td>
<td>Cases negative for macrocapsular invasion</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>
| Evaluation of the results of immunostaining for HER-2/neu:
Table (3): HER-2 expression in the studied cases.

<table>
<thead>
<tr>
<th>Score of Her-2 immunoreactivity</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score 0 (negative)</td>
<td>2 (5%)</td>
</tr>
<tr>
<td>Score 1 (negative)</td>
<td>13 (32%)</td>
</tr>
<tr>
<td>Score 2 (moderately positive)</td>
<td>11 (28%)</td>
</tr>
<tr>
<td>Score 3 (strongly positive)</td>
<td>14 (35%)</td>
</tr>
<tr>
<td>Total number</td>
<td>40 (100%)</td>
</tr>
</tbody>
</table>

The positive immunoreactivity was seen in 18 cases of the clear cell subtype (Figs. 3, 4) in 2 cases of either of the chromophobe and papillary subtypes and finally in one case of each of the collecting duct, sarcomatoid and unclassified subtypes.

Table (4): Correlation between the expression of HER-2/NEU and the different pathological prognostic factors.

<table>
<thead>
<tr>
<th>Prognostic Factors</th>
<th>HER-2/NEU expression</th>
<th>Cases negative for HER-2/NEU expression</th>
<th>Cases positive for HER-2/NEU expression</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor stage</td>
<td>pT1</td>
<td>pT2</td>
<td>pT3 pT4</td>
<td>40</td>
</tr>
<tr>
<td>G I</td>
<td>2</td>
<td>GII</td>
<td>G III G IV</td>
<td></td>
</tr>
<tr>
<td>Macrocapsular invasion</td>
<td></td>
<td>Cases positive for macrocapsular invasion</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Microcapsular invasion</td>
<td></td>
<td>Cases negative for macrocapsular invasion</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Macrovascular invasion</td>
<td></td>
<td>Cases positive for macrocapsular invasion</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Microvascular invasion</td>
<td></td>
<td>Cases negative for macrocapsular invasion</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>
Fig. (1): Renal cell carcinoma, clear cell subtype, with strong positive cell membrane immunoreactivity for EGFR, score 2, (x 400).

Fig. (2): Renal cell carcinoma, clear cell subtype, with moderate positive cell membrane immunostaining for EGFR, score 1, (x 200).

Fig. (3): Renal cell carcinoma, clear cell subtype, with strong positive continuous cell membrane immunostaining for HER-2, score 3, (x 200).

Fig. (4): Renal cell carcinoma, conventional subtype, with faint interrupted cell membrane immunoreactivity for HER-2, score 1, (x200).

Discussion

Renal cancer is the 7th leading malignant condition among men and the 12th among women [15]. Therefore, several studies have focused on evaluating indicators of biologic aggressiveness of RCC.

High expression of EGFR has been associated with advanced stage, poor prognosis and high metastatic potential in many human tumors. The association between EGFR expression and prognosis in RCC has not been established [16]. Considering the set of therapeutic tools targeting EGFR, there are at present two well-identified emerging categories of drugs, with monoclonal antibodies (mAbs) on one hand and tyrosine kinase inhibitors (TKIs) on the other. Both treatment tools have reached an advanced stage of clinical development [6].

As regards EGFR expression in renal cell carcinoma, the current study showed that 22 cases (55% of total cases) were negative for EGFR immunoreactivity (score 0), 12 cases showed moderate positive EGFR cell membrane immunoreactivity (score 1) and 6 cases showed strong positive EGFR cell membrane immunoreactivity (score 2) with a total of 18 positive cases (45% of total cases).

This result disagreed with the majority of studies conducted by other authors as; Mock et al. [17] who reported Thirty-eight cases (76%) expressing strong cell membrane immunoreactivity for EGFR, and Kallio et al. [16] who reported 73% of tumors to be positive for EGFR immunostaining, and Langer et al. [18] who reported membranous EGFR immunostaining in 123 of 132 (93%) of primary and 49 of 53 (92%) of metastatic RCC, and Michael et al. [3] who reported expression in 82.5% of cases, and Ravaud et al. [7] who reported EGFR expression in up to 95% of renal cell carcinoma, and Cohen et al. [19] who found membranous EGFR overexpression in 38 of 44 cases (93.2%), with strong staining (score 2) in 35 cases (79.5%) and finally Pu et al. [13] who found positive EGFR expression in 81% of renal cell carcinoma cases.

The histological heterogeneity of renal cell carcinoma & different methods of immunohistochemical evaluation of EGFR expression, might have led to these different results, strengthening the need for standardization, especially against a background of rapidly evolving EGFR targeted cancer treatment strategies. The small sample size in the current study and the possible racial, genetic and geographic characteristics of Arabs may be an important factor.
Statistical analysis for a possible correlation between EGFR expression and the various prognostic factors in this study (gross capsular invasion, gross vascular invasion, tumor stage, nuclear grade, microcapsular invasion and microvascular invasion) had revealed non significant correlation between both groups.

This result was similar to that reached by Langer et al. [18] who reported that overall EGFR immunoreactivity and intensity of membranous staining were not associated with unfavorable prognosis, and agreed with Michael et al. [3] who demonstrated that positive expression of EGFR was not associated with neither a better nor a worse prognosis, and also with Ravaud et al. [7] who found that the EGFR pathway was probably not a major pathway in RCC development.

However this result disagreed with Mock et al. [17] who reported a tendency toward a shortened survival for EGFR positive tumors and showed that tumor growth fraction (Ki-67 labeling index) was significantly higher in EGFR positive tumors than in EGFR negative tumors, suggesting that rapid tumor proliferation might be responsible for poor prognosis associated with EGFR positive RCC, and disagreed with Cohen et al. [19] who stated that EGFR receptor correlated with pathologic stage but not Fuhrman nuclear grade, overall survival or disease recurrence and also disagreed with Kallio et al. [16] who stated that membranous EGFR positivity was associated with good prognosis.

These heterogeneous results casted doubt on the suggestion by most authors, that activation of EGFR signaling pathway plays an important role in tumorgenesis of renal cell carcinoma and that the patients might benefit from drug therapy targeting EGFR in cases showing expression of such receptor.

Cancers that show HER-2 amplification generally have a poor prognosis and shorter overall survival. HER-2 amplification is also a predictive marker of responsiveness to selected forms of therapy [10]. In clinical usage, HER-2 is important as the target of the monoclonal antibody trastuzumab (Herceptin). Trastuzumab is effective only where the HER-2 receptor is overexpressed [20].

The percentage of positivity was higher than that observed by Zhang et al. [21] who studied 70 patients with RCC and demonstrated HER-2/NEU overexpression in 40% of the cases, and also higher than that stated by Seliger et al. [22] who conducted immunohistochemical analysis of human kidney tumour lesions using 2 HER-2/NEU-specific antibodies and found HER-2/NEU over-expression in more than 40% of primary epithelial renal tumours and more than 30% of primary renal cell carcinoma specimens. Michael et al. [3] as well found positive HER-2 immunoreactivity in only 10% of renal cell carcinomas and Latif et al. [23] examined 27 tumours from renal cell carcinoma patients and found that gene amplification and protein overexpression of HER-2/NEU in renal cell carcinoma was absent.

The discrepancy between the results in current study and all the others, may be attributed to different sample sizes, the different histological subtypes of RCC with different behaviors and genetic makeup, or to the use of different antibody preparations and different techniques. Again, the possible racial, ethnic and geographic characteristics of Arabs may be an important factor.

Statistical analysis for a possible correlation between HER-2 expression and the various prognostic factors in this study (gross capsular invasion, gross vascular invasion, tumor stage, nuclear grade, microcapsular invasion and microvascular invasion) had revealed non significant correlation between both groups.

This result agreed with Rotter et al. [24] who reported low level of expression of HER-2 in renal cell carcinoma and concluded that HER-2 gene might be inversely related to tumor differentiation, but it was probably not involved in progression of RCC, in contrast to carcinomas of other locations, and also agreed with Seliger et al. [22] who found that immunohistochemical analysis of kidney tumors revealed a distinctive pattern of HER-2/NEU expression in RCC but not associated with disease stage or grade, and as well agreed with Latif et al. [23] who examined 27 tumors from renal cell carcinoma patients and found that gene amplification and protein overexpression of HER-2/NEU in renal cell carcinoma was absent and thus not related to disease progression, and finally agreed with Phouc et al. [25] who analyzed multiple biologic markers to identify strong prognostic markers for disease-specific survival of patients with clear cell renal cell carcinoma and found that HER-2 expression was not related to survival.
However, many other studies analyzing HER-2/NEU overexpression have found the opposite; that HER-2/NEU overexpression was associated with a poor prognosis, Zhang et al. [21] studied 70 patients with RCC and demonstrated HER-2/NEU over expression in 40% of the cases and concluded that there was a significant association between HER-2/NEU overexpression and tumor stage in RCC. On the contrary, Michael et al. [3] found that HER-2/NEU overexpression was associated with an increased survival time when compared to the cases that stained negatively for HER-2/NEU, although the difference was not statistically significant.

All these heterogeneous results casted doubt on the suitability of Herceptin in management of patients with renal cell carcinoma and thus the exact role of HER-2/NEU in the pathogenesis of renal cell carcinoma remains unclear. Considering the fact observed in the current study, that HER-2 was consistently and strongly expressed in the non neoplastic renal tubular epithelium, it might be suggested that the pathway of receptor activation in the process of neoplastic transformation may be either over-activated or suppressed. This mandated that further studies must be conducted in order to establish a positive or negative relation between HER-2 receptor expression and renal cell carcinoma.

References
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