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The EGFR Protein Expression and the Gene Copy Number Changes in Renal Cell Carcinomas

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Background : The epidermal growth factor receptor (EGFR) is known to be involved in many tumor promoting activities. EGFR inhibition has been tried as a therapeutic modality in many human malignancies. **Methods :** The expression of EGFR protein and the gene copy number changes were studied in 135 clear cell carcinomas and 16 papillary renal cell carcinomas (RCCs), and these tumors were diagnosed between 1995 and 1997. **Results :** An EGFR protein expression (2+ and 3+) was found in 54.1% of the clear cell RCCs and in 43.8% of the papillary RCCs. In the clear cell RCCs, its expression was associated with male gender, the tumor size ($\geq$4 cm) and high T stages (T2 and T3), with statistical significance. Trisomy and polysomy of the *EGFR* gene were found in 27 (25.7%) and 40 (38.1%) of 105 clear cell RCCs, respectively. Trisomy and polysomy were correlated with an EGFR protein expression and a high clinical T stage, with statistical significance. Among 15 papillary RCCs, 13 tumors showed trisomy (86.7%) and one showed polysomy (6.7%). Amplification was not found in both the clear cell and papillary type RCCs. **Conclusions :** A considerable numbers of RCCs showed an overexpression of EGFR protein and increased *EGFR* gene copy numbers, yet the clinical significance of conducting a FISH study in RCC patients seems to be limited.

Key Words : Renal cell carcinoma; Epidermal growth factor receptor; Fluorescence *in situ* hybridization; Ploidies

Renal cell carcinoma (RCC) is the most common malignancy of the kidney in adults,[1] and radical and partial nephrectomies are the standard treatments for local ized tumors. However, the survival rate still remains low for advanced RCC, despite of great efforts to develop effective therapies, such as chemotherapy, radiation therapy, immunotherapy, and hormone therapy.[2] The epidermal growth factor receptor *(EGFR)* gene is located on chromosome 7p12, and it encodes a 170 kDa membrane glycoprotein. After ligand binding, the EGFR induces the formation of receptor homo-and heterodimers and the activation of the intrinsic tyrosine kinase domain. This activated domain initiates a cascade of events and leads to cell cycle progression, inhibition of apoptosis, angiogenesis, promotion of invasion and metastasis, and other tumor promoting activities.[3] The EGFR overexpression in human cancer has been observed in breast, bladder, prostate and non-small cell lung cancers and glioblastoma.[4] EGFR has recently attracted much attention because of the development of clinical therapies that target this receptor, and the overexpression of EGFR and polysomy 7 have been described in primary RCCs.[3,5]

This study was conducted to detect the frequency of the expression of EGFR protein and the gene number changes of *EGFR*, and to evaluate their correlation with the known clinicopathologic parameters and the survival of patients with RCCs.

MATERIAL AND METHODS

Patients

The tissue microarray (TMA) slides and clinicopathological features used in this study were obtained by the Urological Pathology Study Group of The Korean Society of Pathologists, and these slides included 135 clear cell RCCs and 16 papillary type RCCs that were diagnosed between 1995 and 1997.[6] The clinopathological features included the patients' age and gender, the tumor size, multifocality, the histological type, the presence of necrosis and a sarcomatoid component, Fuhrman's nuclear grade, the modality of treatment and the duration of follow up. The tumor type was histopathologically determined according

to the guidelines of the Union Internationale Contre le Cancer/American Joint Committee on Cancer (UICC/AJCC) and the Heidelberg classification.[7] The papillary RCCs were subgrouped as type 1 and type 2 by the morphologic features of the cells that covered the papillary structures.[8,9] Sarcomatoid differentiation was characterized by areas with fascicles of undifferentiated spindle cells with pleomorphic nuclei and a collagenous matrix.[10] Clear cell RCCs were graded as grade 1 to 4 according to the Fuhrman nuclear grading system, which is based on the size and contour of the nuclei and the conspicuousness of the nucleoli.[11] The tumors were staged and divided into three groups according to the T staging in the TNM system (T1 tumors are limited to the kidney with a size less than 7 cm, T2 tumors are limited to the kidney with a size over 7 cm and T3 tumors are confined to Gerota's fascia of the kidney).[12]

Construction of tissue microarrays

Representative paraffin tumor blocks were selected according to the primary evaluation of the hematoxylin and eosin (H-E)-stained slides before they were prepared for the TMA. Two tissue cores were taken from each of the clear cell RCC, and three tissue cores were taken from each of the papillary RCC, and one core was taken from each block of normal kidney for a control. The cores were placed in a new recipient paraffin block that ultimately contained 72-96 tissue cores. Each array block contained both tumor and control tissue samples. Multiple sections (5 μm thick) were cut from the TMA blocks and then mounted onto microscope slides. The H-E-stained sections of the TMA were reviewed to confirm the presence of representative areas of the tumor.

Immunohistochemical staining

Immunohistochemical studies were performed using a standard two-step method as described in the Dako EnVision system protocol (Dako Corp., Carpinteria, CA, USA). In brief, the TMA slides were deparaffinized in xylene and then rehydrated in a series of graded alcohol solutions. Antigen retrieval was performed by protein digestion with proteinase K, and the endogenous peroxidase activity was blocked with 3% hydrogen peroxidase for 10 min. After washing with distilled water for 5 min, the non-specific binding was blocked with normal horse serum for 20 min at room temperature, and then the slides were incubated for 30 min at room temperature with anti-EGFR antibody (1:100; Dako Corp.). After washing with 0.1% Tris-buffered saline (TBS) for 3 times for 5 min each time, the secondary antibody (ChemMateTM EnVision+/HRP Rb & Mo; Dako Corp.) was added and this was incubated for 30 min at room temperature. The slides were washed again with TBS 3 times for 5 min each time and then 3,3′ diaminobenzidine was applied as a chromogen substrate for 5 min. After washing for 5 min, the sections were counterstained by Harris hematoxylin for 30 s. The slides were dehydrated and coverslipped.

Evaluation of staining intensity

The EGFR immunohistochemical staining intensity of each sample was scored using a four-tier system; negative (no discernible staining or background type staining), 1+ (definite cytoplasmic staining with or without equivocal discontinuous membranous staining), 2+ (unequivocal membrane staining with moderate intensity) and 3+ (strong and complete membrane staining) (Fig. 1).[2,3] To evaluate the association between the expression of EGFR and the clinicopathologic parameters of RCC, the expression of EGFR was divided in to a group with 0 and 1+ and a group with 2+ and 3+.

Fluorescence in situ hybridization (FISH)

FISH was performed by using a Zymed SP · T-Light® EGFr Amplication Probe (Zymed Inc., South San Francisco, CA, USA), according to a published protocol. In brief, the 4 μm TMA sections were heated in a 60˚C oven, and then they were deparaffinized by two successive, 5 min washes in xylene and this was followed by two washes in 100% alcohol. After drying and treatment in 0.2 N HCl for 20 min and washing in SSC washing buffer twice for 3 min for each time, the slides were treated in the pretreatment solution at 80˚C for 30 min, and then they were treated in 0.2% pepsin/protease solution for 20 min at 37˚C, and two washes in SSC washing buffer for 5 min each time. The slides were left in 10% buffered formalin for 10 min, and two washes in SSC washing buffer were done. The DNA was denatured by treatment in SSC/70% formamide twice for 5 min each time at 72˚C. After dehydration with using cold graded alcohol solutions, 10 μL of the probe solution was placed on a tissue section with a coverslip. The slides in the hybridization mixture were put in a heat block for 5 min at 73˚C, and then they were hybridized at 37˚C for 16-18 h. Posthybridization washing was carried out according to the manufacturer's protocol. The tissue sections were counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride and p-phenylenediamine in phosphate-

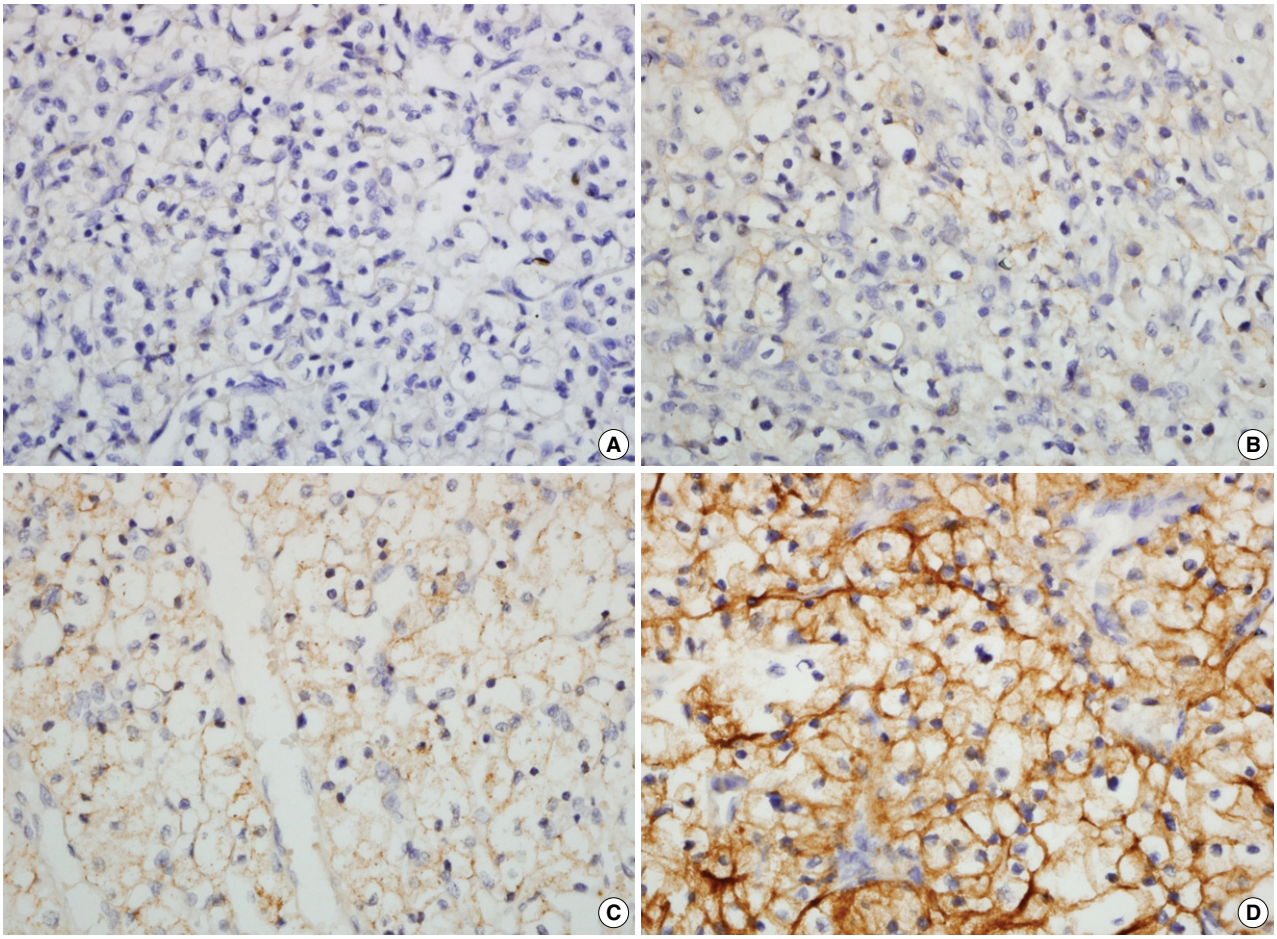

Fig. 1. The immunohistochemical staining for EGFR protein is scored as 0 (A), 1+ (B), 2+ (C), and 3+ (D).

buffered saline and glycerol (DAPI II) and they were examined with a fluorescence microscope (Olympus, Tokyo, Japan) equipped with the Triple Bandpass Filter set for DAPI II, Spectrum Orange™ and SpectrumGreen™, and the filter sets specific to SpectrumOrange and SpectrumGreen. The cells were scored as positive for amplification when a definite cluster or more than 10 orange signals were found. If the cells contained 3 centromeric and EGFR signals, then they were classified as trisomy, whereas if more than 3 centromeric and EGFR signals, then they were equally present, then the cells were considered as polysomy. FISH images were taken using a photographic camera and they were saved as graphic files (Fig. 2).

Statistical analysis

For intervariable assessment, the comparison of the clinicopathological features between the clear cell and papillary type RCCs was carried out using t-tests. The crosstab test was used for evaluating the correlation of the EGFR and FISH results with the clinicopathological features of the clear cell and papillary RCCs. The survival analysis was carried out using the Kaplan-Meier test. Differences were considered to be significant for p-values<0.05. All the statistical analyses were performed using the software SPSS 14.0.1 for Windows (SPSS Incorporation, Chicago, IL, USA).

RESULTS

Comparison of the clinicopathological features

The clinicopathological features of the patients with clear cell and papillary RCCs are summarized in Table 1. The patients with papillary RCCs (46.8 years) were younger than the patients with clear cell RCCs (55.4 years) (p=0.002). Papillary RCCs also demonstrated more frequent necrosis (75.0%) than did the clear

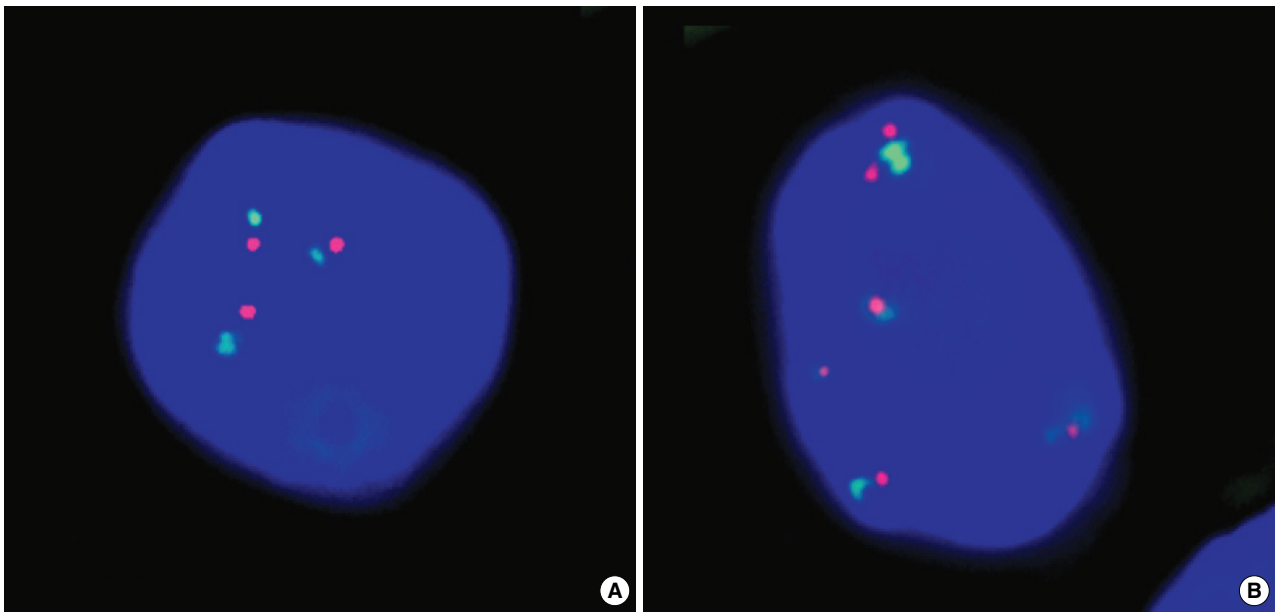

Fig. 2. FISH study with chromosome 7 centromeric probe (green) and *EGFR* gene probe (red) shows trisomy (A) or polysomy (B), but no amplification in renal cell carcinoma.

Table 1. The clinicopathological features of the patients with clear cell and papillary RCCs

Clinicopathological features	Clear cell RCC	Papillary RCC	Statistical significance
No. of cases	135	16	
Age (mean, years)	55.4	46.8	**p=0.002**
Sex			
Male	104 (77.0%)	12 (75.0%)	p=0.855
Female	31 (23.0%)	4 (25.0%)	
Size (cm)[a]	5.78	7.29	p=0.079
Multifocality	13 (9.6%)	1 (6.3%)	p=0.659
Necrosis	43 (31.9%)	12 (75.0%)	**p=0.001**
Sarcomatoid change	8 (5.9%)	0 (0%)	p=0.317
T stage			
T1	64 (47.4%)	6 (37.5%)	p=0.452
T2	45 (33.3%)	4 (25.0%)	
T3	26 (19.2%)	6 (37.5%)	
Fuhrman grade			
1	14 (10.4%)		
2	80 (59.3%)		
3	36 (26.7%)		
4	5 (3.7%)		

[a]The tumor size was evaluated in 129 clear cell and 15 papillary RCCs.
RCC, renal cell carcinoma.

cell RCCs (31.9%) (p=0.001). The papillary RCCs were classified as type 1 in 6 cases (37.5%) and type 2 in 10 cases (62.5%). During follow-up, 8 of the 135 patients with clear cell RCCs (5.9%) died, and the time from radical nephrectomy to death ranged from 5 to 55 months (mean 23.8 months), whereas 4 of 16 patients with papillary RCCs (25.0%), all type 2, died of RCCs during the follow-up period, and the time from operation to death ranged 12 to 43 months (mean 25.5 months). On the survival analysis, the patients with clear cell RCCs showed longer survival than did the patients with papillary RCCs (p=0.008).

EGFR protein expression

Informative TMA results were obtained from 135 clear cell RCCs and 16 papillary RCCs. A membranous expression of EGFR with or without a cytoplasmic expression of EGFR was present in 118 (87.4%) of the 135 clear cell RCCs, and in 11 (68.8%) of the 16 papillary RCCs. However, the comparison of an EGFR expression with the clinopathologic features was done between the tumor group with no or a 1+ expression and the tumor group with a 2+ or 3+ expression.

Clear cell RCCs

The comparison between the expression of EGFR and the clinicopathological features for the clear cell RCCs is shown in Table 2. The evaluation according to tumor size was possible in 129 clear cell RCCs. An EGFR expression of 2+ and 3+ was more commonly found in the tumors with multifocality (61.5%), tumor necrosis (55.8%), sarcomatoid change (75.0%), and Fuhrman grade 3 and 4 (63.4%) than in the tumors with no or a 1+ EGFR expression, but this was without statistical signifi-

Table 2. The comparison between the expression of EGFR and clinicopathological features in clear cell renal cell carcinomas

	No. of cases	EGFR 0+1	EGFR 2+3	Statistical significance
Age				
<50 years	38	22 (57.9%)	16 (42.1%)	p=0.081
≥50 years	97	40 (41.2%)	57 (58.8%)	
Sex				
Male	104	43 (41.3%)	61 (58.7%)	**p=0.05**
Female	31	19 (61.3%)	12 (38.7%)	
Size				
<4 cm	35	22 (62.9%)	13 (37.1%)	**p=0.031**
≥4 cm	94	39 (41.5%)	55 (58.5%)	
Multifocality	13	5 (38.5%)	8 (61.5%)	p=0.570
Necrosis	43	19 (44.2%)	24 (55.8%)	p=0.782
Sarcomatoid change	8	2 (25.0%)	6 (75.0%)	p=0.221
T stage				
T1	64	37 (57.8%)	27 (42.2%)	**p=0.009**
T2 and T3	71	25 (35.2%)	46 (64.8%)	
Fuhrman grade				
1+2	94	47 (50.0%)	47 (50.0%)	p=0.150
3+4	41	15 (36.6%)	26 (63.4%)	

EGFR, epidermal growth factor receptor.

cances. An EGFR expression of 2+ and 3+ was associated with male gender (58.7%, p=0.05), a large tumor size (≥4 cm, 80.9 %, p=0.031) and high T stages (T2 and T3, 64.8%, p=0.009) with statistical significances. On the survival analysis, there was no correlation between the EGFR expression and the patients' survival.

Papillary RCCs

The comparison between the expression of EGFR and the clinicopathological features for the papillary RCCs is shown in Table 3. The information on tumor size was missing in one case. An EGFR expression of 2+ and 3+ was more commonly found in type 2 (60.0%) than in type 1 (16.7%), but this was without statistical significance. There was no association of an EGFR expression with the other clinopathologic parameters.

FISH analysis

FISH was done for 115 clear cell RCCs and 16 papillary RCCs. The relationships of the chromosome 7 copy number changes and the clinicopathological features for the clear cell RCCs are summarized in Table 4.

Clear cell RCCs

The analysis of chromosome 7 was possible in 105 out of 115 RCCs (91.3%). The analysis failed due to loss of cores in 5 cases,

Table 3. Comparison between the expression of EGFR and the clinicopathological features in papillary renal cell carcinomas

	No. of cases	EGFR 0+1	EGFR 2+3	Statistical significance
Age				
<50 years	8	5 (62.5%)	3 (37.5%)	p=0.614
≥50 years	8	4 (50.0%)	4 (50.0%)	
Sex				
Male	12	6 (50.0%)	6 (50.0%)	p=0.383
Female	4	3 (75.0%)	1 (25.0%)	
Size				
<4 cm	3	2 (66.7%)	1 (33.3%)	p=0.605
≥4 cm	12	6 (50.0%)	6 (50.0%)	
Multifocality	1	1 (100%)	0 (0%)	p=0.362
Necrosis	12	6 (50.0%)	6 (50.0%)	p=0.383
T stage				
T1	6	4 (66.7%)	2 (33.3%)	p=0.515
T2 and T3	10	5 (50.0%)	5 (50.0%)	
Type				
Type 1	6	5 (83.3%)	1 (16.7%)	p=0.091
Type 2	10	4 (40.0%)	6 (60.0%)	

EGFR, epidermal growth factor receptor.

Table 4. The relationship of chromosome 7 copy number changes and the clinicopathological features in clear cell renal cell carcinomas

	No. of cases	Disomy	Trisomy+ Polysomy	Statistical significance
Age				
<50 years	29	12 (41.4%)	17 (58.6%)	p=0.494
≥50 years	76	26 (34.2%)	50 (65.8%)	
Sex				
Male	81	27 (33.3%)	54 (45.8%)	p=0.263
Female	24	11 (45.8%)	13 (54.2%)	
Size				
<4 cm	25	13 (52.0%)	12 (48.0%)	p=0.096
≥4 cm	75	25 (33.3%)	50 (66.7%)	
Multifocality	10	3 (30.0%)	7 (70.0%)	p=0.668
Necrosis	37	11 (29.7%)	26 (70.3%)	p=0.310
Sarcomatoid change	6	1 (16.7%)	5 (83.3%)	p=0.305
T stage				
T1	45	22 (48.9%)	23 (51.1%)	**p=0.019**
T2 and T3	60	16 (26.7%)	44 (73.3%)	
Fuhrman grade				
1+2	68	27 (39.7%)	41 (60.3%)	p=0.310
3+4	37	11 (29.7%)	26 (70.3%)	
EGFR expression				
0+1	39	22 (56.4%)	17 (43.6%)	**p=0.001**
2+3	66	16 (24.2%)	50 (75.8%)	

EGFR, epidermal growth factor receptor.

and no signals in another 5 cases. The evaluation according to tumor size was available in 100 cases. Disomy was seen in 38 cases (36.2%), trisomy was seen in 27 cases (25.7%), and polysomy was seen in 40 cases (38.1%). No amplification was found.

Trisomy and polysomy were more commonly found in the tumors with an EGFR expression of 2+ and 3+ (75.8%) than in the tumors with no or a 1+ expression of EGFR (43.6%) (p= 0.001). Trisomy and polysomy were also more frequently seen in the tumors with a high T stages (T2 and T3, 73.3%) than in the T1 tumors (51.1%) and this was statistically significant (p= 0.019). Trisomy and polysomy were more commonly seen in the tumors with multifocality (70.0%), necrosis (70.3%) and sarcomatoid changes (83.3%) than the tumors with disomy, but there was no statistical significance. The tumors with trisomy and polysomy were also more frequently seen in the tumors with high Fuhrman grades (3 and 4, 70.3%) than in the tumors with low Fuhrman grades (1 and 2, 60.3%), but this was without statistical significance. However, on the survival analysis, there was no correlation of trisomy and polysomy with the patients' survival.

Papillary RCCs

FISH analysis was possible in 15 out of the 16 papillary RCCs (93.8%). One specimen failed to show signals. Except for one disomy (6.7%) and one polysomy (6.7%), the remaining 13 cases (86.7%) were trisomy. Amplification was not seen in any cases. Because of the high rate of trisomy in the pRCCs, there was no statistical meaning of the correlation between an EGFR protein expression and the other clinicopathological parameters.

DISCUSSION

EGFR is a transmembrane glycoprotein that is known to be involved in various types of cancer. The mechanism implicated in a cancer's tumorigenesis is specific for each cancer type.[4,13] Mutation in the EGFR gene is one of mechanisms involved in human cancers; mutations in the extracellular domain or the intracellular domain are frequent in glioblastoma multiforme, whereas mutation in the intracellular tyrosine kinase domain is common in non-small cell lung cancer. Defective receptor down-regulation and the cross-talk with other EGFR members are also mechanisms that are involved in cellular transformation. The increased production of the EGFR ligands, namely, TGF-alpha and EGF, leads to dysregulated EGFR activation in various cancers. Dysregulated EGFR activation is often associated with the overexpression of EGFR. Overexpressed EGFR forms spontaneous dimerization and this causes constitutive receptor activation. An EGFR overexpression has been shown to be associated with the amplification of EGFR gene, yet the increased promoter activity or dysregulation at the translational and post-translational levels are also causes of EGFR overexpression. There are two approaches to abolish overexpressed EGFR in cancer treatment, and they are administering tyrosine kinase inhibitors and monoclonal antibodies. For both, the treatment is known to be more effective in the patients with amplified EGFR genes and/or an amplified mRNA expression.[14] Thus, the selecting the patients, who will receive benefit from EGFR-targeted therapy is important.

In addition, the overexpression of EGFR itself has been found to correlate with decreased survival for the patients with various cancers, such as breast, head and neck, hepatocellular, urinary bladder, ovarian, cervical, gastric, colorectal, esophageal and prostatic cancers, and glioblastoma.[15]

The expression rate of EGFR ranged from 47% to 93%.[3,9,16-18] Its significance has been reported to be heterogenous; the membranous EGFR expression has been associated with a poor, good or same prognosis,[3,19,20] while the cytoplasmic expression has been associated with a high tumor grade and stage.[3,9] In our study the membranous EGFR expression in clear cell RCCs was correlated with male gender, the clinical T stage and the tumor size, but not with patients' survival. These results were partly consistent with the studies of Langner *et al.*[3] who reported the correlation of an EGFR expression with the tumor grade, size and stage and patients' survival, and Cohen *et al.*[16] reported an association with the tumor grade and stage, but not with survival.

Although there have been several studies on EGFR mRNA overexpression,[21-23] studies on EGFR gene amplification have not been reported. Amare *et al.*[5] investigated 40 cases of RCCs for polysomy 7, and they reported 100% polysomy in the papillary RCCs and in 56.2% of the clear cell RCCs. Polysomy 7 was correlated with a higher tumor grade, stage and proliferation index, but not with an EGFR expression. In our study using both chromosome centromeric and *EGFR* gene probes, no amplification of the *EGFR* gene was found in all the examined RCCs. Instead, polysomy, including trisomy, was found in 93.4% of the papillary RCCs and in 63.8% of the clear cell RCCs, which was equivalent to the study of Amare *et al*. The increased gene copy number of chromosome 7 was associated with a higher T stage and the overexpression of EGFR protein in the clear cell RCCs.

From this study, it was concluded that a considerable number of the RCCs diagnosed in Korea showed the overexpression of EGFR protein and increased *EGFR* gene copy numbers, and these factors were associated with poor biologic indicators, but not with patients' survival. However, because the overexpression

of EGFR protein was not associated with gene amplification, the clinical significance of EGFR study in RCC patients seems to be limited.

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