UNIVERSITY OF SASSARI
DEPARTMENT OF BIOMEDICAL SCIENCES
INTERNATIONAL PHD SCHOOL IN BIOMOLECULAR AND BIOTECHNOLOGICAL SCIENCES
PROTEOMICS, METABOLOMICS, CLINICAL BIOCHEMISTRY AND CLINICAL MOLECULAR BIOLOGY
XXVI CYCLE
Director: Prof. LEONARDO A. SECHI

ANALYSIS OF EGFR KINASE-DEPENDENT AND KINASE-INDEPENDENT ROLES IN CLEAR CELL RENAL CELL CARCINOMA

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INTRODUCTION

Epidermal Growth Factor Receptor is a tyrosine kinase receptor that is overexpressed in several tumors, and constitutes an ideal molecular target for new biologic therapeutic agents, as in lung and colo-rectal carcinomas [1]. The treatment with EGFR targeted therapies is always related to peculiar genetic abnormalities, as gene mutations and/or increased gene copy number. Clear cell renal cell carcinoma (CCRCC) has been widely investigated for EGFR protein expression, and previous studies on wide series of CCRCC demonstrated that EGFR protein expression is a common occurrence in CCRCCs, ranging from 50 to 90% of positive cases in different experiences [2]. So far, clinical trials data showed that EGFR-targeted molecular therapies are not effective for CCRCC. Since EGFR gene abnormalities are apparently not appreciable in CCRCCs, EGFR overexpression should be related to alterations in the post-translational regulation machinery, with anomalous protein stabilization or defective receptor downregulation, increasing its ligand-mediated activation.

Nevertheless, since EGFR overexpression in CCRCC has been stated, further investigations on EGFR kinase and non-kinase roles is mandatory for widening the therapeutical adjuvant strategies for these tumors.

Epidemiology of Renal Cell Carcinoma

Renal cell carcinoma (RCC) is recognized as a heterogeneous group of tumors originating from the epithelium of renal tubules, which display distinctive genetic/molecular backgrounds, unique morphological features and characteristic clinical courses. The majority of kidney cancers (80-85%) are renal cell carcinomas (RCCs) arising from the renal parenchyma. The remaining 15- 20% are for the most part urothelial carcinomas of the renal pelvis [3].

RCC is the third most common lethal urological malignancy after prostate and bladder cancers [4]. It represents 3% of tumors in adults with an age-standardised rate incidence of 5.8 and mortality of 1.4 per 100,000, respectively, in more developed areas [5].

The incidence presents geographical variations, and it is generally highest in Europe, North America, and Australia. The lowest rates are reported in Japan, Asia, Africa and China. This variability suggests a strong role of exogenous factors in addition with geographic differences in genetic susceptibility and diagnostic variability [6,7].

Incidence worldwide per year is about 209,000 new cases and 102,000 patients die from this disease. In 2008, in Europe, 88,300 patients were diagnosed with RCC, and 39,230 died from the disease [8].

According to Italian Cancer Registry, in Italy during the period 2003-2005, kidney cancer was the eighth most common malignancy in male population, representing 3.8% in the overall incidence of tumors in men and 2.5% in women. The median incidence rate is 26.4 cases per 100,000 men and 13.4 cases per 100,000 women per year, which corresponds about 9,800 cases per year [9].

The incidence rate showed an increase among men and a substantial stability among women. This tumor has a North-South gradient with lower incidence in the Central and Southern regions. There is a 2:1 predominance of men over women, with the peak incidence occurs between the ages of 60 and 70 [9].

Approximately 20–30% of patients are diagnosed at the metastatic stage of disease and half of the remaining patients will experience recurrence after an initially curative treatment. The overall 5-year survival rate of RCC is estimated in the range of 50%, whereas long-term survival decreases in case of metastatic disease and does not exceed 10% [5].

In the last few years, the incidence rate variation has been influenced by a greater use of diagnostic imaging, which has improved the diagnostic accuracy and increased the percentage of early diagnosis, thus expanding the therapeutic opportunities.

Etiology and Risk Factors

The information on risk factors for renal cell cancer was established by a large number of case-control studies and recently supported by cohort studies [6]. Several risk factors for the development of RCC have been identified. No single risk factor explaining the development of all renal cell cancers has been found. Cigarette smoking, obesity, hypertension-antihypertensive medication and family history are the major risk factors identified for the development of renal carcinoma [7].

In heavy smokers, the risk is up to 2-fold compared to nonsmokers. Mutagens have been found in the urine of smokers, and the excretion correlate to the tar content [10,11].

About obesity, the risk increases with an increased body mass index, both in men and women. It has been suggested that changes in levels of steroid-hormones, insulin like growth factor-1, cholesterol, vitamin D, adipose tissue derived cytokines and hormones, all obesity-related events, are involved in cancer development [12, 13, 14]. However, data on the prognostic impact of overweight in RCC is still conflicting.

Hypertension increases the risk of Renal Cell Carcinoma, while the antihypertensive drugs such as diuretics are not independent risk factor [15].

The majority of renal cell cancers occur in sporadic form, whereas hereditary renal cancers account for a small proportion of the patients. A family history of disease is been associated with an increased risk of 4 times. The major hereditary forms include von Hippel Lindau syndrome, hereditary papillary renal cancer syndrome, RCC associated with hereditary leiomyomatosis syndrome, Birt-Hogg-Dubè syndrome and constitutional chromosome 3 syndrome. Recently, a rare form of familial renal cancer related to hyperparathyroidism-jaw tumor syndrome has been described.

Each of the inherited syndromes predisposes to distinct types of renal carcinoma, which tend to be multiple and bilateral, and present at an earlier age [16, 17].

The role of other risk factors, such as analgesics, exposure to asbestos, gasoline or trichloroethylene, as well as the potential protective effects of alcohol, fruit and vegetables, have to be taken into consideration in RCC oncogenesis. An association between renal cell cancer and disorders of the pre-existing kidney and urinary tract, including kidney stones and infections, has been proposed. These associations are statistically significant and have been confirmed in many studies [6, 18].

Clinical Features

Many renal masses are asymptomatic and non-palpable until the later stages of the disease. The classic triad of symptoms, consisting of flank pain, gross haematuria and a palpable abdominal mass, only occurs in 6-10% of cases [19]. 20% of RCC presents with systemic signs of advanced disease and among these, the most frequent are: declining weight up to cachexia, chronic low-grade fever, anemia, metastatic bone pain, chronic cough from pulmonary involvement. During the course of the tumor in 10-40% of cases, a paraneoplastic syndrome may be evident, which usually regresses after the removal of the tumor. Paraneoplastic syndromes result from the humoral release of various tumor-associated proteins which are directly produced by the cancer cells or by the immune system in response to the tumor. Patients can suffer from symptoms due to metastases. The most frequent sites for RCC metastases are lung parenchyma, bone, liver and brain, but RCCs can metastasize to virtually any organ site [20].

Classification of renal cell carcinomas

The classification of renal neoplasia has undergone significant changes over the last 3 decades due to the large variety of histological subtypes and the continuous evolution of molecular methods. It is therefore difficult to create a simple and appropriate classification.

The RCCs have historically been classified according to the cell type present (clear, granular, oncocytic or spindle) and according to the pattern of growth (acinar, papillary, or solid); this classification criteria provided, however, limited information on clinical behavior of the cancer itself. A further histopathologic classification, proposed in 1986, was based on morphological, histochemical and ultrastructural tumor characteristics, and wass supported by cytogenetic and molecular analysis.

Today these tumors are classified according to the system drawn up in 2004 by the World Health Organization (WHO), which describes categories and entities based on morphology, immunoreactivity and genetic features, in the light of new genetic alterations highlighted by the discoveries of recent years. Advances in understanding of basic morphology, immunohistochemistry, cytogenetics, and molecular pathology have led to an expansion in the number of distinct tumor entities which are currently recognized[21] .

According to WHO 2004 classification, renal malignant tumors include those with the highest incidence, such as clear cell RCC, papillary RCC, chromophobe carcinoma, and collecting duct carcinoma of Bellini, and less frequent variants, as multilocular cystic RCC, carcinoma associated with Xp11 translocation / 2TFE3, carcinoma associated with neuroblastoma, mucinous tubular and spindle cell carcinoma. Oncocytoma, papillary adenoma, and metanephric adenoma are recognized as renal benign neoplasms [21].

Clear cell renal cell carcinoma (CCRCC) represents the wide majority of renal malignant neoplasms, accounting for approximately 70–80% of cancers [22].

Among less frequent neoplastic entities, papillary RCC represents 10-15% of kidney cancers and has a less aggressive clinical course than CCRCC. It has a tendency to be multifocal and bilateral and consists of epithelial cells arranged to form papillae and tubules in variable proportions. Microscopically the papillae contains a delicate fibrovascular core with aggregates of foamy macrophages, cholesterol crystals and frequent hemosiderin granules. In 1997, two morphological types have been described: the type 1 contain a single layer of small cells with little cytoplasm covering the underlying fibrovascular cores, whereas the type 2 is constituted by pseudo-stratified cells with more eosinophilic cytoplasm and higher nuclear grade. The prognosis of type 1 is more favorable than that of type 2. The commonest genetic alterations are trisomy or tetrasomy of chromosome 7, trisomy of chromosome 17 and loss of chromosome Y. Trisomy of 12, 16 and 20 is also found in PRCC and may be related to tumor progression, while loss of heterozygosity at 9p13 is associated with shorter survival [21, 22].

Chromophobe carcinoma accounts for 3–5% of RCCs and it is mostly sporadic, although some cases are related to the Birt-Hogg-Dubé (BHD) syndrome. Macroscopically it shows a demarcated beige or pale-tan color mass without capsules. Microscopic examination reveals 2 types. The typical variant is characterised by huge pale cells with reticulated cytoplasm and prominent cell membrane. The eosinophilic variant is purely composed of intensively eosinophilic cells with prominent cell membranes. The cells have irregular, often wrinkled, nuclei. Some are binucleated. Nucleoli are usually small. Perinuclear halos are common. The underlying genetic abnormality is monosomy in chromosomes 1, 2, 6, 10, 13, 17 or 21[21, 22].

Collecting duct or Bellini's duct carcinoma represents less than 1% of RCCs. It is characterized by irregular channels lined by a highly atypical epithelium, which can have a hobnail appearance. Renal medullary carcinoma is considered a subtype of collecting duct carcinoma and associated with

the sickle cell trait. Less than 3% of RCCs are characterized by indeterminate histological features and they are called unclassified or undifferentiated RCCs [21, 22].

Some studies have shown that CCRCC has the worst prognosis compared to papillary and chromophobe variants. This concerns especially organ-localized tumors. When stage and nuclear grade were included in the analyses, tumor type lost independent prognostic significance. However, the multilocular cystic variant of CCRCC and mucinous tubular and spindle cell RCC have favorable prognosis. Collecting duct and medullary carcinoma have poor prognosis. For all the major subtypes sarcomatoid dedifferentiation is associated with adverse prognosis [21, 22].

Recently, the International Society of Urological Pathology (ISUP) Vancouver Classification of Renal Neoplasia proposed new epithelial neoplasms: tubulocystic renal cell carcinoma (RCC), acquired cystic disease-associated RCC, clear cell (tubulo) papillary RCC, the MiT family translocation RCCs (in particular t(6;11) RCC), and hereditary leiomyomatosis RCC syndrome-associated RCC. In addition, 3 rare variants were considered as emerging or provisional new entities: thyroid-like follicular RCC; succinate dehydrogenase B deficiency-associated RCC; and ALK translocation RCC [23].

CLEAR CELL RENAL CELL CARCINOMA

CCRCC is the most common type of renal cancer that occur in adults who are older than 40 years of age and there is a 1,5:1 predominance of men over women [24]. It presents a mostly silent development and, for this reason, the diagnosis is made late in relation to the stage of the disease. The clinical onset is manifested by the classic triad of symptoms consisting of hematuria, back pain and an abdominal palpable mass. Other signs and symptoms of the disease frequently can be summarized as: anemia, liver dysfunction, weight loss, hypoalbuminemia, malaise general, hypercalcemia, and anorexia. Nevertheless, many patients are asymptomatic and the tumor, usually is diagnosed incidentally during abdominal imaging.

Gross pathology

Macroscopically, the tumor presents a bright yellow color due to the accumulation of lipids in malignant cells, and fibrosis, often at the center of the lesion, can impart a gray color [25]. The size of the tumor may vary from a few millimeters up to dimensions such as to reach a few kilograms in weight. Hyalinization and fibrosis are common in tumors in the early stage, while in advanced tumors coagulation necrosis can be observed [26]. The tumor is commonly well circumscribed, although the capsule is often absent [22]. A low percentage of cases presents cystic change and calcification or ossification. Bilateral tumors were found in 0.5-3% of patients, and are more frequent in patients with Von Hippel Lindau syndrome and tuberous sclerosis [25].

Microscopic features

The microscopic analysis of CCRCC shows a proliferation of large cells, with well-defined cell membranes, and a clear, optically empty cytoplasm, caused by the removal of lipids by routine histological procedures. The tumor may also contain a variable proportion of cells with a residual granular eosinophilic cytoplasm, around the nucleus, but these are rarely the predominant cell type in the tumor. The nuclei appear round, oval and fairly regular in well-differentiated forms, where mitotic

activity is absent or rare, while they may show irregularities and hyperchromia with atypical mitotic figures in the most aggressive forms. CCRCC may progress into a sarcomatoid carcinoma, where a higher grade of cytological atypia is usually recognizable, even with multinucleated giant cells, and morphologic signs of dedifferentiation are apparent.

Hyaline globules can be appreciable both outside and inside the cells [25]. From the ultrastructural point of view, the cells have long microvilli, reminiscent the brush border of the proximal renal tubules, while the Golgi apparatus and the rough endoplasmic reticulum tend to be scarce or absent, and may be pushed toward the cell periphery by the accumulation of lipids and glycogen [27].

The architecture of the tumor can be either alveolar, tubular and microcystic. In the alveolar pattern, the alveoli are rounded and separated by a light network of highly vascularized connective tissue whose blood vessels have a thin wall. In the tubular pattern, neoplastic epithelial cells are aligned to define a central lumen. In areas of microcystic degeneration, is generally present necrotic material and red blood cells, and cysts are separated by fibrous septa and irregular in shape and size. This type of architecture characterizes the rare variant known as multilocular cystic clear cell carcinoma [25]. The presence of poorly differentiated components of sarcomatoid or rhabdoid type is associated with a higher clinical aggressiveness [28].

Genomic Alteration

CCRCC is characterized by a considerable molecular heterogeneity which results in an altered cell proliferation, cell death, metabolic activity and tumor microenvironment. This suggests that the traditional prognostic factors such as pathologic staging, grading according to Fuhrman and nuclear morphology may not be sufficient for a correct characterization and classification of these tumors. Therefore, it is difficult to predict the progression of the disease, the therapeutic response and survival. Over the past decade, the introduction of techniques as "new generation sequencing" has led

to significant progress in understanding the molecular pathogenesis of CCRCC by identifying specific genetic alterations and molecular mechanisms related to them. However, it remains a challenge still open the identification of prognostic and predictive biomarkers [29].

The most frequent genomic alteration detected in CCRCC is the loss of short arm of chromosome 3 at position 3p25 associated with von Hippel-Lindau (VHL) tumor suppressor gene inactivation that leads to a lack of functional VHL protein (pVHL). So it confirmed the critical role of VHL gene in CCRCC development. Over 800 VHL mutations were identified in both hereditary and sporadic CCRCC and more than 50% of them are frameshift and nonsense mutations which produce loss of pVHL function[29, 30] .

In familial forms, germ line mutations are followed by mutation, methylation, or loss of the remaining wild-type VHL allele in the tumor. Sporadic forms are characterized by somatic biallelic inactivation of the VHL gene. 80% of sporadic CCRCC displays alterations of the VHL gene which involve either somatic mutations, loss of heterozygosity and promoter hypermethylation but the effective biallelic inactivation of VHL is found in 60–70% of CCRCC cases [31-35]. Moreover, 30–40% of sporadic CCRCCs have a functional VHL gene, suggesting that different genes and other pathways are involved in the tumor pathogenesis.

The VHL gene is an evolutionarily well conserved tumor-suppressor gene composed of at least three exons. VHL gene coding for a protein of a 30-kDa (pVHL) constituted by 213 amino acids with multiple functions.

The best investigated function is its role as a substrate recognition component of a multiprotein E3 ubiquitin protein ligase complex involving proteins such elongins B and C Cul2 and Rbx1, that target other protein for ubiquitination and subsequent proteasomal degradation [36]. The hypoxia-inducible factor (HIF), the principal target, is a transcription factor that mediates adaptive responses to hypoxia. It enhances O2 delivery and promotes cell survival under a low oxygen environment by

upregulating numerous genes. HIF-1 is a heterodimer consisting of a hypoxia inducible alpha subunit and a constitutively expressed beta subunit [37-39].

HIF-1α is upregulated in the post-translational level through a decrease in protein degradation in response to hypoxia (O2-dependent mechanism). HIF-1α is also induced in the translational level, namely, by upregulation of its synthesis in response to growth factor stimulations and subsequent activations of phosphoinositol-3-kinase (PI3K)/Akt/ mammalian target of rapamycin (mTOR) or RAF/mitogen-activated protein kinase kinase (MEK)/mitogen-activated protein kinase (MAPK) pathways (O2-independent mechanism) [40].

Under normoxic conditions, pVHL binds the hydroxylated hypoxia inducible factor (HIF) alpha which leads to its ubiquitination and degradation [38].

Under hypoxic conditions or in the absence of functional VHL , the stabilization of HIFα, which is not degraded, leads to the formation of a heterodimer with HIF1β which initiates enhanced transcription of HIF target genes VEGF, EGF, PDGF, erythropoietin, glucose transporter 1, involved

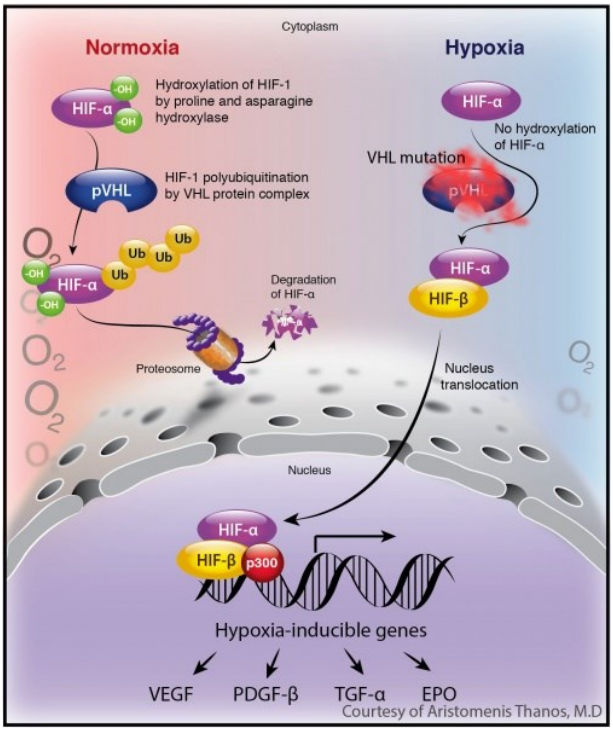


Figure 1. VHL-HIF Pathway

in the regulation of cellular processes such as angiogenesis, cellular metabolism and cell growth (Figure 1).

Both HIF1α and HIF2α show common but also distinct transcription patterns. HIF1α preferably drives the expression of genes important for apoptotic and glycolytic pathways, whereas HIF2α activates genes involved in cell proliferation and angiogenesis. The inactivation of VHL resulting in overexpression of HIF may be involved in the development of cancer through deregulation of cellular processes described above [38].

The CCRCC can be classified according to the different mutational status and expression of the VHL and HIF genes, into three distinct CCRCC phenotypes tumors: the first with the gene wild-type VHL, and the absence of expression of HIF1α and HIF2α (VHL+, HIF1α-, HIF2α-); the second with mutated VHL with increased expression of both HIF1α that of HIF2α (VHL-, HIF1α+, HIF2α+); the third VHL deficient tumors with only overexpression of HIF2α (VHL-, HIF1α-, HIF2α+) [41]. Studies have shown that HIF-1 α inhibits transcription of the c-Myc oncoprotein, whereas HIF-2 α potentiates c-Myc transcriptional activity and cellular proliferation. Then both molecules oppositely regulate oncogene activation in CCRCC tumorigenesis, resulting in distinct gene expression patterns that correlate with differences in clinical outcomes. Another study has shown significant differences in gene expression profiles of VHL deficient CCRCC tumors. VHL +/+ cancers exhibit a specific gene expression pattern that may represent a separate CCRCC subset, which is not able to respond to VHL/HIF pathway targeted therapy [42].

Consequently, gene expression heterogeneity of CCRCCs clearly reflects differences in disease course, drug response variability among patients, tumor drug resistance, and therapy effectiveness.

Furthermore, a recent study, using NotI-microarrays (NMA), a sensitive technology which allow to identify both genetic (deletions/amplification) and epigenetic (methylation/demethylation) changes simultaneously, identify epigenetic and genetic alterations of 22 genes located on

chromosome 3 in CCRCC. Among them there are well-known tumor suppressor gene and candidate (VHL, CTDSPL, LRRC3B, ALDH1L1 and EPHB1) and genes that were not previously considered involved in cancer development (LRRN1, GORASP1, FGD5, and PLCL2). These genes encodes proteins which are involved in signaling pathways and biological functions frequently affected during progression of different tumor, however a part of the gene and their protein functions are still unknown. These results suggest that methylation on chromosome 3 is a common event in CCRCC [43].

Recently a systematic large-scale sequencing of more than 3500 genes in 101 CCRCC identified new molecular markers correlated with clear cell renal cell carcinoma; these genes encode enzymes that demethylate (UTX, JARID1C) or methylate (SETD2) key lysine residues of histone H3. The epigenetic modifications of histone H3 regulate chromatin structure thereby influencing the control of gene transcription, in CCRCC such epigenetic mutations can be identified in only 15% of cases. PBMR1 gene mutations were found in 50% of tumors analyzed; the gene maps to chromosome 3p21 and encodes the protein BAF180 representing the subunits of complex PBAF SWI / SNI that targets the chromatin remodeling it. This protein complex is involved in the regulation of replication, transcription, DNA repair, and control of cell proliferation and differentiation. Several studies identify the gene PBMR1 as a "gatekeeper" of CCRCC, suggesting a potential role for therapeutic options [39] . The high incidence of mutations in the PBRM1 gene (50% of cases) were confirmed in independent CCRCC sets, which makes the PBRM1 gene a second major mutated gene in CCRCC (following VHL) [44, 45]. Moreover, loss of PBRM1 protein expression correlates with clinical parameters, including advanced tumor stage, low differentiation grade and, worse patient outcome [46]. Mutations in the BAP1 gene, a protein associated with BRCA, were identified in 15% of clear cell renal carcinomas, indicating that mutations in the BAP1 gene and the gene PBRM1 are mutually exclusive. Other studies have demonstrated the presence of amplification of chromosome 8q resulting

in overexpression of c-MYC gene. Finally, the RASSF1A gene is hypermethylated and inactivated in 30-50% of sporadic cases of clear cell renal cell carcinoma and papillary, while rarely found gene mutations.

The second most frequent chromosomal alteration is the duplication of chromosome 5q but the oncogenes located in this region remain unknown. Several studies indicate that the candidate genes mapped to 5q chromosome are TGFB1 amplified in 70% of CCRCC cases and CSF1R upregulated in 30% of tumors [47, 48].

Other common polysomy are those of chromosomes 7 (18-30%), 16 (11%), 12 (10-15%), 20 (10%), and 2 (9-14%). During the progression of the disease, the cancer cells can also express the entire polyploidy karyotype, in turn, associated with more losses and gains of chromosomal material transcription, DNA repair, control of cell proliferation and differentiation [49].

Prognostic Categorization

In assessing the prognosis of CCRCC, we take into account several clinical-pathological features, such as pathological staging according to the TNM system (tumor, node, metastasis), where T stands for tumor size, N for lymph node status and M for the presence of metastases; Fuhrman nuclear grade, which correlates with tumor aggressiveness; the presence of necrosis.

To date, different prognostic models to predict survival and identify patients with a high risk of recurrence, have been proposed. The SSIGN Score, a model that integrates stage, size of primary tumor, nuclear grade and presence of necrosis in a single score, has proven particularly useful for predicting the '"outcome" of the patient. In fact, higher SSIGN score are correlated with reduced tumor-specific survival in patients with clear cell renal cell carcinoma.

In a multivariate retrospective analysis of 600 patients with metastatic renal cell carcinoma Elson et Coll have identified five indicators of survival: ECOG PS score (Eastern Cooperative Oncology Group Performance Status), the period of time between diagnosis and the first systemic

treatment, the number of metastatic sites, previous systemic therapy and weight loss. On the basis of these factors, the authors have stratified patients in five group characterized by different survival.

The UCLA Integrated Staging System (UISS system), developed by Zisman et al, is valid for patients with metastatic RCC and with localized tumors [50].

It is a model that integrates the TNM staging, ECOG PS, and Fuhrman grade and categorize patients into three groups (low, intermediate, and high risk) to predict overall survival. In an international multicenter this system has not been confirmed to be reliable and accurate for metastatic disease therefore retains its prognostic value only in localized disease [51].

The Memorial Sloan Kettering Cancer Center (MSKCC) prognostic system was proposed by Motzer et al, who analyzed a series of 670 patients with RCC in advanced stage and treated with immunotherapy and chemotherapy, identifying five pretreatment factors significantly correlated with a worse prognosis: low Karnofsky Performance Status (KPS) , high lactate dehydrogenase (LDH) and serum calcium , low hemoglobin and presence/absence of nephrectomy [52].

Motzer, using these variables, stratified patients into the following groups: favorable, intermediate, and unfavorable risk with different prognosis, survival ranged from 20 months for the group with a good prognosis, 4 months for a poor prognosis. A subsequent validation of the Motzer prognostic system has shown that the majority of patients fall within the intermediate-risk group and no account is taken of independent prognostic factors such as the previous radiation treatments and the number of metastatic sites. The addition of these two parameters allowed to redistribute patients initially considered to be at intermediate prognosis and classify them in the group with a poor prognosis.

Motzer et al. also have developed a nomogram which should be able to predict the probability of disease progression at 12 months after start of treatment, using parameters that are an evolution of Motzer prognostic criteria [53].

New prognostic markers

The wide application of immunohistochemical and molecular techniques has allowed to identify new potential prognostic markers and their potential prognostic impact. They are represented by adhesion molecules, as E-Cadherin-6; molecules induced by hypoxia, as VEGF, HIF, CAIX; molecules stimulating immunosuppressive responses, as B7-H1 and B7-H4; and anti-apoptotic molecules, as surviving [54, 55].

VEGF is a polypeptide overexpressed in the tumor, which has been associated with a decreased survival time and it is correlated with tumor size, grading, necrosis, stage and microvessel invasion. Increased production of VEGF is relate to VHL gene alterations [56, 57]. HIF is a typical tumor marker, and its correlation with the survival of patients with CCRCC has been extensively studied; in fact, HIF1α is overexpressed in all renal neoplasms, but the clear cell subtype is the one with the largest share of expression; HIF2α is also overexpressed in the epithelium of numerous tubular renal tumors, instead both HIF1α that HIF2α, in at least 50% of the cases, are not expressed in sarcomatoid component of the tumor [58, 59]. Multivariate analysis demonstrated that patients with HIF1α expression and sarcomatoid differentiation showed higher PFS and cancer-specific survival (CSS) rate. On the contrary HIF2 α expression was not associated with survival [55].

Carbonic anhydrase IX (CAIX) is currently one of the factors most studied and is a transmembrane protein, involved in the regulation of both intracellular and extracellular pH, whose gene expression is modulated by HIF1. Although CAIX is absent in the context of normal renal parenchyma, is overexpressed in 95% of clear cell carcinomas and in 50% of papillary [60]. Some authors have demonstrated the role of CAIX in predicting cause-specific survival of patients with clear cell carcinoma.

B7-H1 and B7-H4 are members of the super-family of immunoglobulins, in particular B7-H1 is a glycoprotein expressed on the cell membrane, which acts as activator of T lymphocytes, and is

correlated to a high degree of mortality in renal tumors, while B7-H4 is expressed in activated T cells [61, 62].

Survivin is an inhibitor of apoptosis protein detectable in all renal tumors, whose overexpression has been associated with adverse tumor features and poor prognosis [63, 64].

Some chromosomal alterations in RCC have been related with prognosis: in fact, the deletion of the short arm of chromosome 3 was associated with more favorable prognosis, while the loss of 4p, 9p, and 14q is associated with poor prognosis.

Treatment

The treatment of renal cell carcinoma has changed dramatically in recent years with the advent of targeted agents.

The therapeutic approach is primarily surgical, in particular in patients with early-stage disease, although about 30% of patients will display tumor recurrence and distant metastases. Depending on the size and extent of the tumor, a partial or total nephrectomy may be performed, and recently new laparoscopic procedures have been developed in order to improve the recovery of the patient.

Chemotherapy, which appears to be active in many solid tumors, resulted in a minimal clinical benefit in patients with renal cell carcinoma because no chemotherapeutic agent shows significant antitumor activity. Currently the indications for chemotherapy in metastatic renal cell carcinoma are limited to specific clinical conditions.

Since RCC is highly resistant to chemotherapy, the treatment is based on the administration of immunomodulatory agents such as interleukin-2 (IL-2) and / or interferon-alpha (IFN-α) with a significant antitumor activity, widely used as first-line treatment of metastatic disease. Response rates with these cytokines are low (5 to 20%), and median overall survival is approximately 12 months.

Interleukin-2 (IL-2) is a cytokine produced by activated T cells. Upon binding with specific receptor on the cell membrane, IL-2 promotes clonal expansion and enhances the cytotoxic activity,

which is in an increase in the proliferation and cytolytic functions of cellular effectors, such as CD8+ cytotoxic T lymphocytes and "natural killer" cells.

The interferons are natural glycoproteins with antiviral, antiproliferative, and immunomodulatory properties. Interferon-alpha (IFN-α) is a protein produced by lymphocytes, lymphoblasts and macrophages which is able to activate macrophages and monocytes, to increase the activity of the 'natural killer' cells, to induce the expression of antigens on the cell surface, and to enhance the activity of cytotoxic T lymphocytes. IFN-α antineoplastic mechanisms are not clear, but an important activity as anti-angiogenic modulator, especially when it is administered at low doses, has been reported.

The percentage of patients showing a complete response, especially with IL-2, was very limited, i.e. approximately 12.4% of patients, and it was often associated with significant toxicity. In addition, it was shown that the benefit of immunotherapy was limited to patients with good prognosis: recent data show a higher response rate in patients treated with high-dose IL-2 and selected on the basis of specific clinics characteristics [65].

Since 2005, new molecules for the treatment of metastatic renal carcinoma have been developed, based on new findings concerning the biological mechanisms responsible for the renal carcinogenesis.

The development of "Targeted Therapies" has enabled to develop more personalized treatment but also improved the clinical outcome of patients with metastatic renal cell carcinoma. The median survival of patients with metastatic renal cell carcinoma treated with cytokines was about 10 months, whereas an improvement in progression-free survival of disease up to 27 months, and an increase in overall survival up to 40 months, has been estimated for targeted treatments.

TARGETED THERAPIES

Recent advances in molecular biology have allowed to study the different expression of genes involved in neoplasms and proteins produced by them in order to determine a detailed molecular profile of tumors.

The resulting clinical and therapeutic application of these studies made it possible to customize therapies based on tumor biomolecular characterization.

The Targeted Therapy is defined as a drug that blocks the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progression.

The major advantage of targeted therapies is the selective action on tumor cells, making them potentially more effective and less toxic, as well as the enhancement of therapeutic effects of chemotherapy and radiotherapy. Moreover, data obtained from clinical research support the possibility of using a wide range of agents directed to different molecular targets. The greater restriction on their use is now represented by spectrum of activity limited to those particular subgroups of tumors sharing the same histologic phenotype, depend on specific molecular alterations. Main mechanisms of action of identified for targeted therapies are: blocking of growth factors and their receptors, modification of the function of proteins involved in the regulation of gene expression or other cellular functions, induction of apoptosis, inhibition of tumor cell 'angiogenesis, enhancement of the immune response against cancer cells and production of toxic molecules against cancer cells (Figure 2).

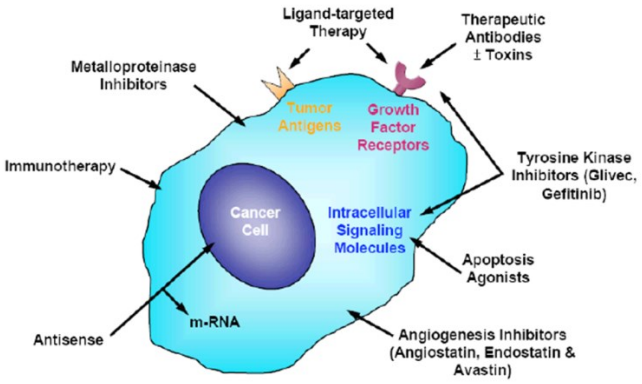


Figure 2. Targeted therapies mechanism of action

Recently, protein kinases, especially protein tyrosine kinase, which catalyse key phosphorylation reactions in signaling cascades affecting every aspect of cell growth, differentiation and metabolism, have become the main therapeutic target in cancer treatment [66].

Most extracellular signals, implicated in the regulation of growth and cell survival, are amplified and transduced into the cell through three processes: the activation of receptor tyrosine kinase , receptors coupled to GTP-binding proteins, or through the cytokine receptors [67]. All these receptors systems, directly or indirectly, involve protein kinases in signal transduction. The development of cancer can be correlated with a wide variety of cellular mechanisms that include the overexpression of growth factors, such as the vascular-endotelial growth factor (VEGF), the transforming growth factor alpha (TGF-alpha), receptors of growth factors (the receptor for platelet-derived growth factor, PDGFR, or receptor for the epidermal growth factor, EGFR), the deregulation of kinase (ABL or EGFR), the deregulation of the activity of kinases by the activation of oncogenes (via signal transduction Ras/Raf), the loss of function of tumor suppressor genes (p53, pRb).

The knowledge of signal transduction pathways and the method by which a cell responds to external signals have made that the protein-kinase, could become an important target for the treatment of cancer, especially in cases where an inappropriate activation of these kinase protein is involved in the pathogenesis and progression of cancer [66.].

Study and comprehension of molecular mechanisms involved in the pathogenesis of tumors, have allowed the identification of markers useful both for diagnosis and as targets for therapeutic treatment. The resulting clinical and therapeutic application of these results is allowing to customize treatments based on biomolecular characterization of the tumor. More and more are using selective drugs that act on single altered molecules (growth factors, receptors, enzymes, etc.), responsible for the growth and uncontrolled spread of cancer cells, their resistance to traditional therapies and angiogenesis.

Among the targets proposed by the new selective therapies, used in the treatment of cancer, there are a number of molecules with antigenic properties, generally localized to the cell membranes and expressed in a different way from the neoplastic elements than normal cells, potentially susceptible to the action of targeted humanized monoclonal antibodies. This therapeutic approach was reflected particularly in the adjuvant treatment of breast cancer, colon cancer, lung cancer and certain lymphoproliferative disorders [68, 69].

Clear examples of their potential efficacy are given by Imatinib-mesylate, which was approved for chronic myeloid leukemia and Gastro Intestinal Stromal tumors (GIST), mesenchymal neoplasms that develop in different sections of the gastrointestinal tract, characterized by the presence in the tumor cells of c-KIT and PDGFR-alpha gene mutations resulting in gain of function of the corresponding receptor. It is known that GISTs are poorly responsive to conventional treatments such as chemotherapy and radiotherapy, and excellent results were recently obtained with Imatinib-mesylate treatment. Reports in the literature indicate that 75-90% of patients with advanced-stage GISTs treated with Imatinib-mesylate show clinical benefits.

Trastuzumab, a humanized monoclonal antibody specific for the human epidermal growth factor receptor type 2 (HER2), a cell-surface tyrosine kinase receptor overexpressed by 25% to 30% of breast cancers, was approved as a single agent in first-line treatment of HER2-Overexpressing

metastatic breast cancer. Patients with HER2 positive Breast Cancer manifested short disease-free and overall survival. A large phase II trial prove that trastuzumab monotherapy is active against HER2-overexpressing breast cancer in patients previously treated with chemotherapy for metastatic disease. Additionally, the results of a phase III trial 7 indicate that trastuzumab significantly enhances the activity of first-line chemotherapy and provides a survival advantage to women with HER2-overexpressing breast cancer.

In 2007, Bevacizumab, a recombinant humanized monoclonal antibody that binds to vascular endothelial factor (VEGF) inducing angiogenesis inhibition, was approved for the treatment of patients with metastatic HER-2 negative breast cancer. The approval was based on a Phase 3 study that showed that Avastin in combination with paclitaxel is able to reduce by 52% the risk of tumor progression or death compared to patients treated only with chemotherapy, doubling-free disease survival. [70]

Another example is Cetuximab, an EGFR inhibitor, utilized for the treatment of non-small cell lung cancer (NSCLS). About 70% of cases of lung cancer shows overexpression of EGFR which correlates with a poor prognosis, EGFR genomic amplification and activating mutations of the receptor.

RCC and Targeted Therapies

Small molecule tyrosine kinase inhibitors (TKIs) were the first targeted therapies approved for the treatment of RCC. They bind to tyrosine kinase receptors located on the intracellular domain of cell surface growth factor receptors, blocking intracellular signaling, and therefore promoting tumor shrinkage.

For the treatment of CCRCC different types of drugs have been developed: the first class includes drugs aimed to inhibit neoplastic angiogenesis, such as Bevacizumab, a monoclonal antibody directed against the vascular endothelial growth factor (VEGF); a second class includes tyrosine

kinase inhibitors (TKIs) such as Sunitinib, Sorafenib, Pazopanib, and Axitinib, in addition to mTOR inhibitors, Everolimus and Temsirolimus (Figure 3). Each of these molecules acts specifically against a component of the intracellular molecular mechanisms that characterize the renal carcinoma. These agents mostly focus on downstream disruption of the VHL/HIF pathway by targeting VEGFR, or inhibiting the mTOR pathway.

VEGF is a potent pro-angiogenic protein, leading to increased vascular permeability and endothelial cell proliferation/migration. Therapeutic inhibition of the VEGF pathway thus has strong biologic rationale in RCC.

Sorafenib, approved in 2005, is a multikinase inhibitor which was designed as a c-Raf and b-Raf inhibitor. The Ras/Raf signaling pathway is a mediator of tumor cell proliferation and angiogenesis.

Sorafenib is a TKI that inhibits VEGFR2 and 3, c-Kit, PDGFR and the serine-threonine kinase BRAF and CRAF (kinase components of the molecular cascade of RAF / MEK / ERK pathway, which is involved in survival / proliferation of cells cancer). Sorafenib is used as a second-line RRC treatment due to improved progression-free survival (PFS) when compared with cytokine therapy. In a clinical phase III trial patients with metastatic RCC, progressing after prior treatment with cytokines, were randomized to receive II line treatment with Sorafenib versus placebo. [71]

The benefit in terms of PFS after therapy with Sorafenib was statistically significant. In a subsequent randomized study phase II comparing Sorafenib vs. IFN-α as treatment of the line, in 189 patients with metastatic RCC, no statistically significant difference between the two drugs were reported. In elderly patients (> 70 years), Sorafenib was effective and safe in the same way as shown for younger patients with a toxicity profile slightly higher in elderly patients, although the difference was not statistically significant.

Sorafenib has been approved in II line therapy for patients with RCC metastatic progression after cytokine therapy or to the line of patients candidates for treatment with cytokines [72]. Oral Sorafenib therapy prolonged PFS in patients with advanced CCRCC in whom first-line therapy had failed. This improvement was associated with an increased number of adverse events, as compared with placebo.

Sunitinib is an oral inhibitor of VEGFR1 and 2, PDGFR alpha and beta, FGFR1, FLT-3, CSF-1R and c-KIT, was approved by the Food and Drug Administration (FDA) for the treatment of RCC, and is recommended as first-line treatment in advanced stages [73]. A randomized phase III trial, Sunitinib to IFN-α in first-line therapy in 750 patients with metastatic RCC, showed a statistically significant advantage for PFS. Sunitinib is very effective in patients with metastatic CCR. Moreover, it has also shown activity in patients with unfavorable characteristics of the disease (2 brain metastases, non-clear cell histology) and in elderly patients [74]

Sunitinib is an oral inhibitor of VEGFR1 and 2, PDGFR alpha and beta, FGFR1, FLT-3, CSF-1R and c-KIT, which has been approved in 2006 by the Food and Drug Administration (FDA) for the treatment of CCRCC, and is recommended as first-line drug in treatment of the advanced stages. Two multicentre phase II trials investigated Sunitinib's efficacy as second-line therapy for patients with a prior nephrectomy and cytokine-refractory RCC [75, 76].

Both trials demonstrated that Sunitinib is active in the second-line setting with overall response rate RRs of 40 and 33% and PFS of 8.7 and 8.8 months .A randomized phase III trial confirmed the results of the phase II studies and compared Sunitinib with IFN-a in 750 patients with untreated metastatic clear cell RCC, showing a statistically significant advantage for PFS. Moreover, it has also shown activity in patients with unfavorable characteristics of the disease (2 brain metastases, non-clear cell histology) and in elderly patients [77].

Pazopanib, targeting VEGFR 1– 3, PDGFR α and β, FGFR1, FGFR3,KIT, and CSF-1R, has more recently been approved for use as a first-line therapy for metastatic RCC. A phase III trial reports that Pazopanib may have a favorable side effect profile as compared to Sunitinib, although hepatoxicity is more frequent with Pazopanib. A randomized, double-blind, phase III study evaluated efficacy and safety of Pazopanib monotherapy demonstrating significant improvement in PFS and tumor response compared with placebo in treatment-naive and cytokine-pretreated patients with advanced and/or metastatic RCC [78].

Pazopanib is likely to be indicated in the treatment of patients with locally advanced or metastatic CCR, with predominant clear cell histology:, in patients with good prognosis / intermediate (according to MSKCC Prognostic Score); in line II, in patients previously treated with cytokines.

Axitinib, a second-generation inhibitor of VEGFR 1, 2, and 3 at sub-nanomolar drug concentrations. It is a small molecule approved by the FDA as a second-line therapy of advanced renal cell carcinoma. Axitinib resulted in significantly longer PFS compared to another multikinase inhibitor, Sorafenib, and was developed to be a more potent and selective inhibitor of VEGFRs [79]. Relative potency of Axitinib is 50–450 times greater than that of the first-generation VEGFR inhibitors. Additionally, first-generation inhibitors block other targets, such as PDGFR, b-RAF, KIT, and FLT-3, which are not inhibited by Axitinib. These off-target activities might contribute to the adverse effects of the first-generation inhibitors, suggesting that more specific inhibitors of VEGFR such as Axitinib might have an enhanced therapeutic efficacy. In a phase 2 study including 13 patients with cytokine-refractory renal cell carcinoma, the objective response rate with Axitinib was 44%, with a median time to progression of 15-7 months and an overall survival of 29-9 months.

Bevacizumab, a monoclonal antibody, given intravenously, that binds to and neutralizes circulating VEGF protein but does not affect the VEGF receptor, approved by FDA for colon cancer in 2004 and for other cancers, including RCC in 2009. This antiangiogenic antibody has produced a

significant prolongation of time to disease progression compared with placebo in patients with treatment-refractory metastatic RCC in a small randomized trial [80].

Bevacizumab it is not approved for RCC monotherapy but in combination with the immune modulator interferon alpha, producing high response rate and PFS exceeding 10 months.

Indeed, a prospective randomized trial demonstrates that addition of Bevacizumab to IFN produces significantly prolonged PFS and a higher ORR compared with IFN monotherapy in patients with untreated metastatic clear-cell RCC [81]. This is the first demonstrations of the benefit of combining multiple non chemotherapy agents in cancer systemic therapy. It is noteworthy that the mechanism of these two agents may not be entirely independent, as IFN has demonstrated antiangiogenic effects and antibody-mediated VEGF inhibition has antitumor effects through improvement in dendritic cell function.

Two agents targeting the mTOR pathway are FDA approved for the treatment of RCC: temsirolimus and everolimus.

Temsirolimus is a derivative of Sirolimus (rapamycin), it is an inhibitor of mammalian target of rapamycin kinase, mTORC1, administered intravenously and approved for first line treatment of RCC. mTOR is a component of RAS/PI3K/PKB intracellular signaling pathways regulating cell growth and proliferation, metabolism, and angiogenesis, which is involved in the cell growth and proliferation, and in the response of such cells to hypoxic stress. Abnormal functioning of the mTOR pathway may contribute to the pathogenesis of renal cell carcinoma (RCC) [65]. Interruption of mTOR signaling decreases levels of HIF, VEGF, and other intracellular factors involved in progression of the cell through its cycle. It is the only drug recommended as a category 1 therapy for RCC patients with poor prognosis.

Everolimus is an immunosuppressant mTOR inhibitor, orally administered, approved by the FDA in 2009 in patients with advanced RCC after progression following treatment with Sunitinib or

Sorafenib. According to a trial involving 410 RCC patients previously treated with inhibitors of VEGF and subsequently with Everolimus, a prolonged PFS compared to placebo was appreciable [82].

It is known that EGFR overexpression is correlated with a poor prognosis in CCRCC, reduced survival and increase risk of metastatic disease. Clinical trials have shown that EGFR inhibitors, when used as single agents, did not demonstrate treatment efficacy in patients with advanced RCC.

Phase II trials, open label study showed that patients with advanced, metastatic, or relapsed RCC did not reveal any objective responses following treatment with Gefitinib (IRESSA), the first EGFR tyrosine kinase inhibitor to be approved for the treatment of cancer [83].

In another study by Drucker et al. involving 18 patients with advanced RCC treated with IRESSA compared with IFN-α, the treatment did not result in any complete or partial responses, and 13 patients (81%) had progression of disease within 4 months after onset of therapy [84].

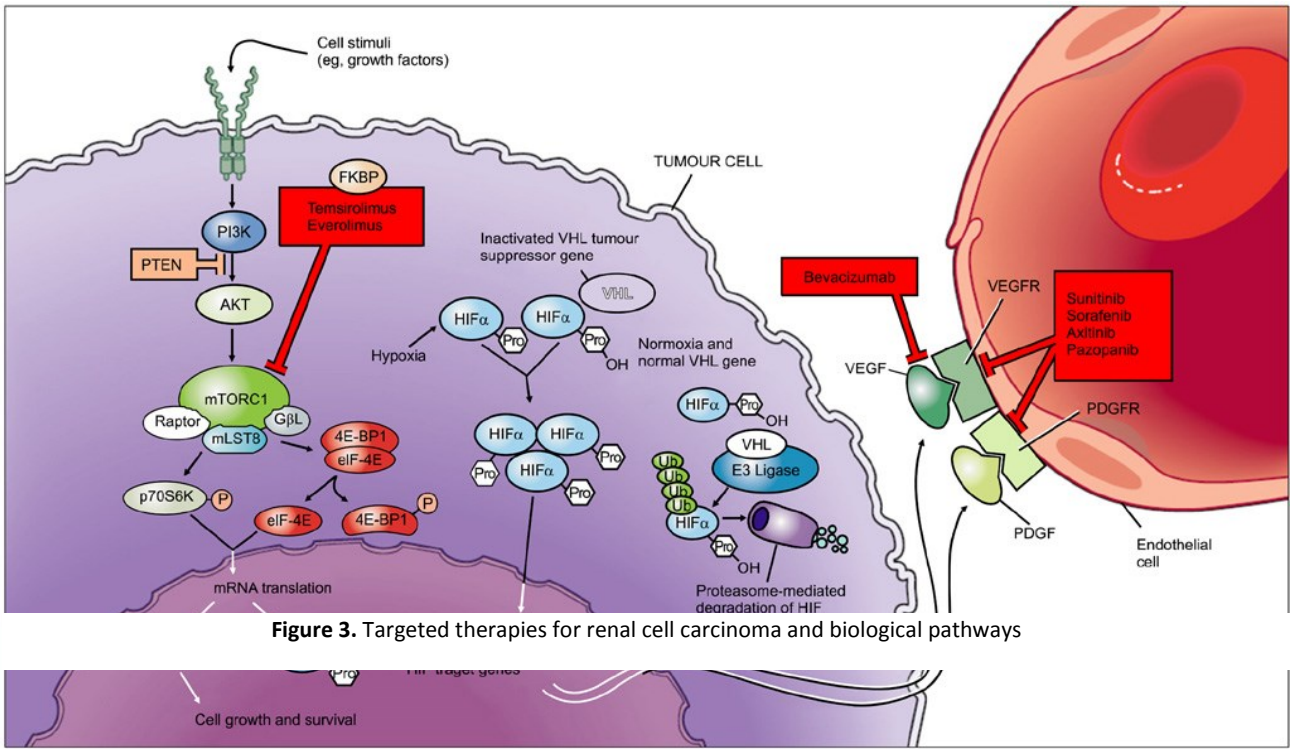


Figure 3. Targeted therapies for renal cell carcinoma and biological pathways

This lack of objective responses in patients with metastatic RCC has also been observed in a phase II trial of the EGFR monoclonal antibody Cetuximab (ABX-EGF), where time to progression was not prolonged with Cetuximab compared with IFN-α data [85]

Neoadjuvant biological therapy is the administration of "targeted" before nephrectomy, with two different objectives: reduce the volume of the primary tumor in patients with CCR to allow partial nephrectomy; reduce the volume of the primary tumor and the extent of metastatic spread in patients with metastatic CCR.

To date, neoadjuvant therapy is a treatment modality currently under study, which is not to be considered, at the moment, in routine clinical practice.

EPIDERMAL GROWTH FACTOR RECEPTOR

Epidermal growth factor receptors represent a large family of tyrosine kinase receptors (TKR) expressed in several types of cancer, including breast, lung, esophageal, and head and neck [86] EGFR and its family members are the major contributors of a complex signaling cascade that modulates growth, differentiation, adhesion, migration and survival of cancer cells. Given their multidimensional role in the progression of cancer, they have emerged as attractive candidates for anti-cancer therapy.

Specifically EGFR is one of the most investigated TKRs, is constitutively expressed in some normal mammalian epithelial cells where has been shown to regulate a variety of important functions, including growth, survival, differentiation, and morphogenesis. It may be used as a model for understanding signal transduction pathways.

The epidermal growth factor receptor (EGFR) is a member of the human epidermal growth factor receptor (HER)-erbB family of receptor tyrosine kinases, which also includes HER2/neu (erbB2), HER3 (erbB3), and HER4 (erbB4) (Figure 4). These receptors are widely expressed in all tissues and they activate a wide range of biological responses including mitogenesis, migration, differentiation, apoptosis and dedifferentiation. [87]

EGFR is a transmembrane protein of 170 kD which comprises a polypeptide chain of 1186 amino acids encoded by a gene that maps to the short arm of chromosome 7.

EGFR consists of an extracellular N-terminal ligand binding domain and a dimerization arm, a lipophilic region transmembrane domain, and an intracellular, highly conserved, cytoplasmic c-terminal tyrosine kinase domain that is able to phosphorylate tyrosine residues on different intracellular adaptor proteins (Figure 5).

The extracellular domain of the receptor is variable and it enables binding to different ligands. It is divided into 4 sub-domains (I-IV): the sub-domains I and III mediate ligand binding, while the

sub-domains II and IV are rich in cysteine residues, are involved in receptor dimerization. The extracellular domain in the absence of ligand is shown as a closed domain. This inactive conformation presents intramolecular interactions between the regions II and IV, preventing the formation of a binding pocket for the ligand. In the presence of ligand, the sub-domains II and IV are opened allowing the sub-domains I and III to form a binding pocket for the ligand, which results in dimerization of the receptor (Figure 6).

The intracellular domain comprises three domains: a juxtamembrane region (JM), a tyrosine kinase domain (kinase) and a carboxy-terminal regulatory region (CT). The JM region has been reported to have a number of regulatory functions including regulation of downstream proteins and ligand-dependent receptor internalisation [88, 89] (Figure 5).

Many different ligands are able to bind to the EGFR extracellular domain such as, epidermal growth factor (EGF), transforming growth factor–alpha(TGF-α), amphiregulin (AR), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EPR), epigen (EPG) and neuregulins 1-6 (NRG). EGF, TGF-α, AR, BTC and EPR bind specifically EGFR and among these EGF, TGF-α are the most important ligands.

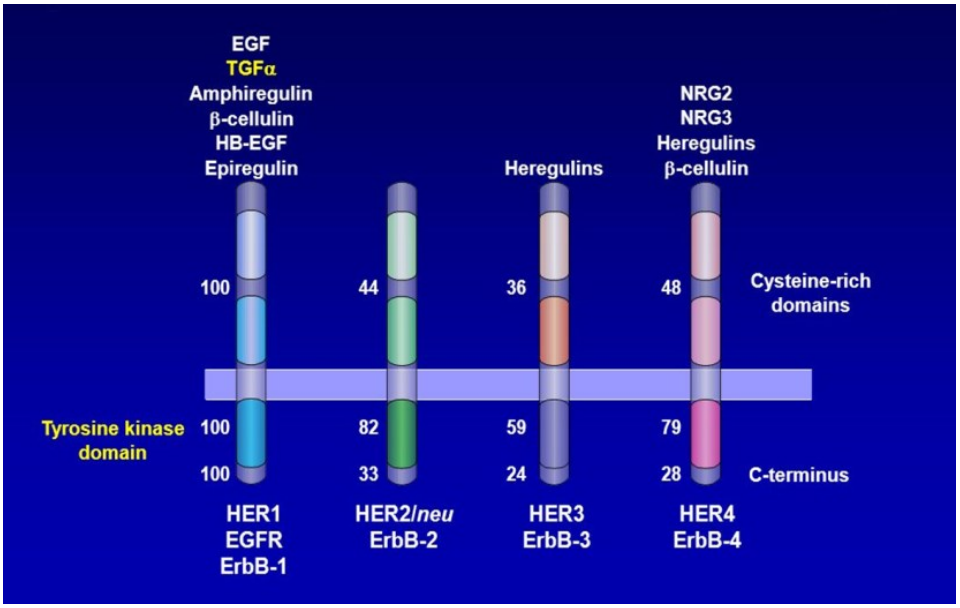


Figure 4. EGFRs family and specific ligands

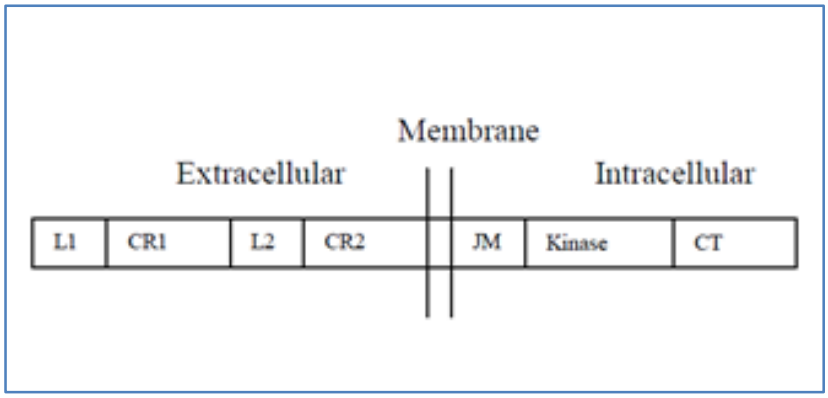


Figure 5. Structure of the EGF receptor

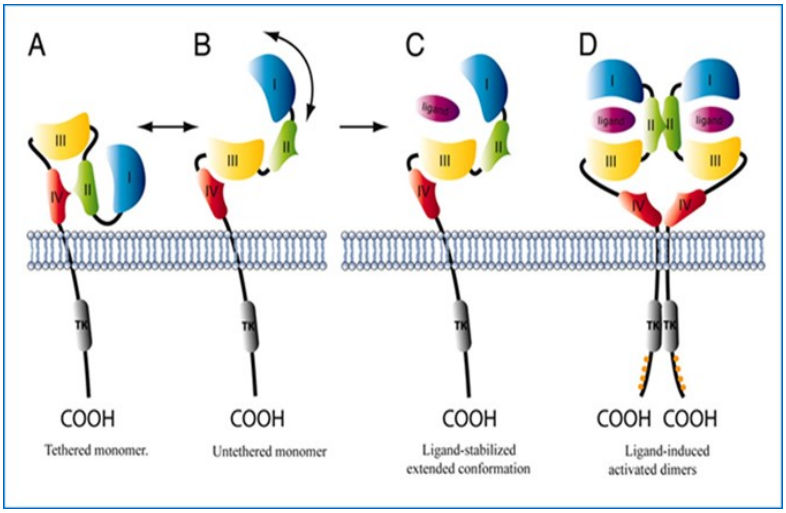


Figure 6. Model for molecular mechanism of ligand-induced EGFR activation.

The stimulating factors (ligands) bind to the extracellular domain causing receptor homo- or hetero-dimerization, thus inducing conformational change of the intracellular phosphorylation components, enabling downstream signaling. The downstream signaling cascades include the RAS-RAF-MAP-Kinase pathway, the phosphatidylinositol 3–Kinase and the Akt pathway. These signaling pathway participate in cell cycle activation, cell survival, proliferation and angiogenesis. EGFR pathway stimulation has been shown to promote tumor cell motility, adhesion, and metastasis [90]

Signal transduction pathways activated by EGFR

The binding of epidermal growth factor or other growth factors to the EGFR determines its dimerization, which is followed by the activation of intracellular protein kinases through phosphorylation, giving, thus, the origin signal transduction. The signal transduction pathways activated by EGFR control different cell functions such as growth, apoptosis, differentiation, and cell migration (Figure 7) [91].

PI3K/Akt/mTOR signalling pathway

Studies demonstrated that the PI3K/Akt/mTOR pathway plays a crucial role in many biological processes including proliferation, differentiation, anti-apoptosis, tumorigenesis and angiogenesis [92]. Furthermore, this pathway has been shown to be involved in the regulation of a variety of cancers including breast cancer [93], lung cancer [94], melanomas [95] and leukaemia [96]

The phosphatidyl-inositol-3-kinase (PI3K) are a family of heterodimeric proteins consisting of a catalytic subunit and a regulatory that phosphorylate PIP2 (phosphatidylinositol) in PIP3 (fosfatidyltriinositolo). The PI3K are divided into three classes (I, II and III) according to protein domains that constitute them and the specificity of the substrate.

Class I PI3K are cytoplasmic heterodimers composed of a catalytic subunit (p110) and an adaptor protein (p85), which contains two SH2 and one SH3 binding domain. The Class I PI3K can be further divided into the Class IA isoforms, which is activated by RTKs, and the Class IB, which is activated by Gβγ subunits of GPCRs. The Class I PI3K are the only ones that are able to convert phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2) to the second messenger phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P3) [92]. Class II PI3Ks, which comprise three catalytic isoforms including C2α, C2β and C2γ, are mainly associated with the phospholipid membranes. This class of PI3K produces PI-3,4,5-P3 from phosphatidylinositol and may also produce PI-3,4-P2 from phosphatidylinositol-4-phosphate (PI-4-P) [97]. The structure of Class III PI3Ks are similar as Class

I, as they are heterodimers composed of a catalytic subunit (Vps34) and a regulatory subunit (p150). They produce PI-3,4,5-P3 through the conversion of phosphatidylinositol [98].

Akt is the most important downstream effector of PIP3 and is involved in the regulation of different physiological processes such as the proliferation, differentiation, apoptosis, transcription, translation and metabolism.

Akt is a serine/threonine protein kinase which belongs to the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A/protein kinase G/PKC super family. In mammals, three members of Akt have been identified, Akt1 [99], Akt2 [100] and Akt3 [101]. Isoforms consist of three domains: an amino terminal pleckstrin homology domain, a central kinase domain and a carboxyl-terminal regulatory domain.

Akt is activated through a double phosphorylation and subsequent recruitment of the protein to the cell membrane. Through its PH domain, present at the N-terminus of the molecule, is in fact able to bind molecules of PIP3 present at the level of the plasma membrane. The recruitment of Akt to the cell membrane causes a conformational change that allows the molecule to its residual thr308 to come phosphorylated by PDK1, a serine / threonine kinase containing a PH domain, while the second phosphorylation, occurs on the serine residue in position 473 to place level of the C-terminal end of the protein complex and is determined by the mTOR (mammalian target of rapamycin) / Rictor (rapamycin-insensitive companion of mTOR) .

Akt is phosphorylated migrates in both the cytoplasm and nucleus, with a prevalence in this second cell compartment since most of its targets have a cytosolic localization.

Given the importance of Akt in control of both proliferation and cell survival, alterations in the PI3K-AKT signaling pathway are a key point in the onset and progression of many cancers. There are many, in fact, human tumors with mutations and chromosomal rearrangements at the level of this pathway.

mTOR is a serine/threonine kinase that regulates various biological effects including cell proliferation, survival, motility, protein synthesis and transcription [102]. The major function of mTOR is the regulation of translation, especially the recruitment of ribosomes to mRNA. mTOR directly or indirectly regulates the activation of several components of the ribosomes recruitment mechanism

Ras/Raf/MAPK pathway

The Mitogen-Activated Protein (MAP) kinases are a group of serine / threonine kinases that mediate many regulatory signals. There are three main groups of MAP kinase: the family of p38 MAP kinase, the family of extracellular signal-regulated kinase (ERK) and the family of kinase N-terminal c-Jun (JNK). The different members of the signal transduction pathway controlling important cellular functions such as cell survival, apoptosis and transcription; their abnormal activation is responsible for malignant transformation and tumor progression. In recent years, numerous studies have established that the route of the Ras/Raf/MAPK is closely linked to via PI3K-Akt; in fact, the activated Ras protein is able to regulate both pathways. [103].

The activation of the MAP kinase cascade and in particular via the Ras/Raf/MAPK, occurs following the binding of growth factors to tyrosine kinase receptor localized on the membrane, which dimerize and interact with G proteins, represented by the Ras for via the ERK and Rho family members by way of JNK and p38. These proteins have GTP-ase activities and promote the phosphorylation of downstream proteins, including RAF, which is recruited to the membrane and phosphorylated. RAF phosphorylates MEK, which is able to always activate ERK by phosphorylation. The latter can activate several transcription factors such as c-MYC and CREB that promote cell proliferation while inhibiting the induction of apoptosis [103].

Recent studies have demonstrated that activation of this pathway is correlated with the development of tumors, including renal cell carcinoma, genetic alterations of different pathway

components were observed with a frequency of about 20%, and in particular 'overexpression' of some of these molecules was observed in 52% of renal cell carcinomas [104]

JAK/STAT pathway

The Signal Transducers and Activators of Transcription (STATs) are a family of cytoplasmic proteins with the function to transduce the signal coming from the extracellular environment to the nucleus; they are activated by phosphorylation of a tyrosine residue located in the C-terminal domain of the molecule; This phosphorylation is mediated by kinases belonging to the Janus family (JAK), which bind to the intracellular domain of the activated epidermal growth factor receptor; in this way is triggered dimerization of STATs, which occurs through the interaction between the tyrosine phosphorylated SH and domains present in these proteins; the homo-and heterodimers formed by STATs subsequently translocate to the nucleus where they regulate gene transcription.

STAT3 is a family member that is activated STATs inappropriately in several forms of cancer. It seems that the continuous activation of STAT3 is sufficient for neoplastic transformation. The role of STATs proteins may depend on both post-translational modifications such as methylation and acetylation, and phosphorylation of serine residues operated by different kinases; impaired activation of the protein may, therefore, reflect both an increased activity of kinases regulating both epigenetic alterations that would alter the transcription of STAT3 [105].

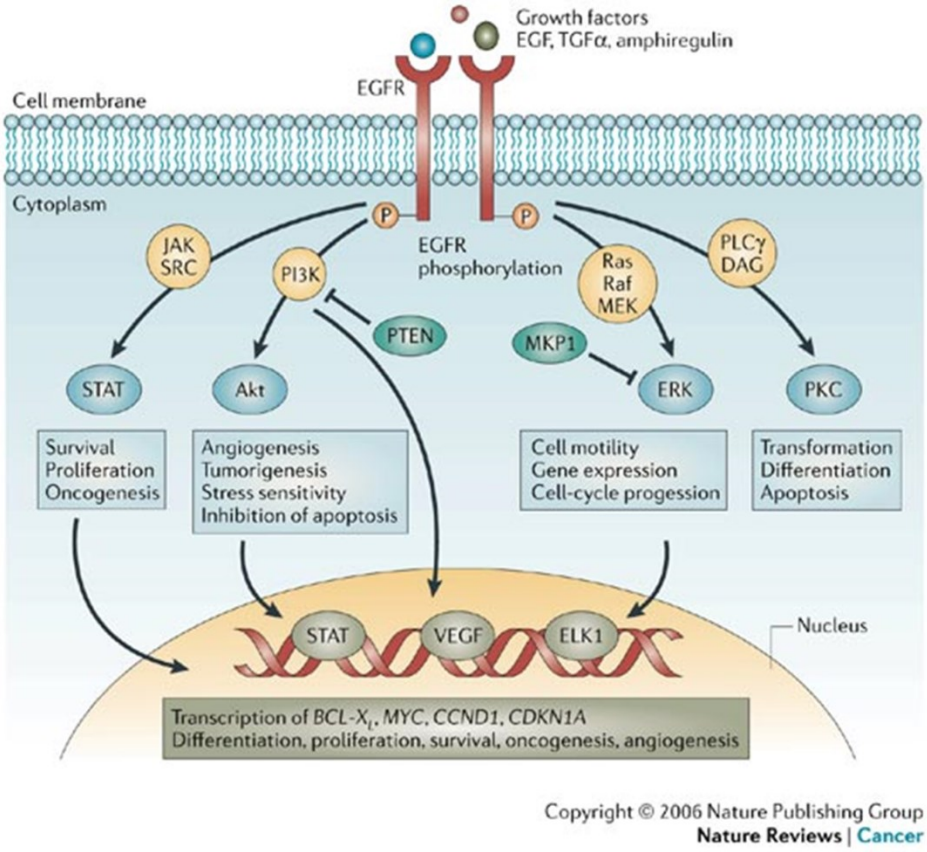


Figure 7. Main downstream signalling pathways regulated by EGFR

The main mechanism of inactivation of EGFR, with a consequent reduction in numbers of the receptor, is represented by lysosomal degradation that follows endocytosis of the receptor-ligand complex. Recently it was shown that c-Cbl, enzyme with ubiquitin-ligase function, bind phosphorylated EGFR and mono-ubiquitylates several lysine residues allowing endocytosis and subsequent lysosome degradation. Mutations of c-Cbl have been identified in several types of malignancies; the mutated protein retains the ability to bind to tyrosine residues phosphorylated receptor tyrosine kinase, but is not able to ubiquitinate the receptor itself [106].

Recent results indicate a predominant role of VHL in the degradation of activated EGFR, the proposed mechanism involves the activation of constitutive HIF which suppresses the expression of rabaptina5, a transcriptional levels, a protein that plays an important role in mediating the fusion of endosomes with lysosomes; the loss of rabaptina5 is therefore responsible for the reduced lysosomal

degradation of the receptor. Moreover, VHL appears to play a dual role in the degradation of activated EGFR. In fact it was shown that the VHL promotes the poly-ubiquitination of EGFR activated, independently of c-Cbl, resulting receptor proteasomal degradation. Therefore, excessive kinase activity of EGFR in renal clear cell carcinomas, resulting in a growth advantage of the tumor, may be caused by failure of receptor inactivation in VHL-deficient tumor cells [107]. Moreover, in clear cell renal cell carcinomas with loss of VHL, HIF overexpression is responsible for an increased transcription of the gene of the transforming growth factor α (TGFα), an agonist of EGFR, and at the same time improves the efficiency of translation of the mRNA of the epidermal growth factor receptor. Increased expression of EGFR, thus, together with the increase in the levels of TGFα, may contribute to the progression of the tumor.

EGFR targeted therapies

Inhibition of the EGFR tyrosine kinase activity has been used for cancer therapy. Several studies conferred significant benefits from anti-EGFR agents in several types of solid tumors including colorectal, head and neck cancer, NSCLC and pancreatic cancer in terms of overall survival, progression free survival and overall response rate [108], however, other types of cancer are resistant to EGFR inhibitors such as Renal cell carcinoma and prostate cancer [109, 110].

The complexity of EGFR-related signal transduction pathways and their importance for cell growth and survival highlights the potential role of EGFR alterations in the development and maintenance of cancer. Abnormalities in EGFR functions are associated will all key features of cancer development and growth, including autonomous cell proliferation, invasion, angiogenic and metastatic potential. Aberrant EGFR signaling can be initiated by several events, such as altered ligand production, receptor mutations or deletions, persistent activation.

EGFR is frequently overexpressed in human tumors such as head and neck squamous cell carcinoma (HNSCC), glioblastoma, NSCL, breast, colorectal, bladder, prostate, ovarian carcinomas.

It has been for a long time the biomarker used for the application of Targeted Therapies. Other frequent alterations identified in the EGFR concern gene mutations that in approximately 90% of cases involve exons 18-24 coding for the tyrosine kinase domain of the receptor, resulting in a gain of function of the receptor. The amplification of the EGFR gene has rarely been shown in human tumors, although fluorescent in situ hybridization (FISH) has identified an increase in the gene copy number in tumor cells in approximately 25-40% of patients with carcinoma of the colon and rectum, lung and head and neck. These data suggest that inhibition of EGFR may be a viable therapeutic option in the treatment of cancer and it must be considered the functional involvement of EGFR in diverse cellular processes.

Gene amplification leading to EGFR overexpression and often accompanied by other structural rearrangements that cause in-frame deletions in the extracellular domain of the receptor, the most frequent being the type III mutated variant of EGFR, denominated EGFRvIII. This mutated receptor is missing exons 2-7, which encode for a part of the dimerization arm and it is characterized by a deletion in the extracellular domain that leads to constitutive activation of its tyrosine kinase domain (Figure 8). EGFRvIII is expressed in tumors like breast, lung and ovarian carcinomas. Moreover, high EGFR expression seems to correlate with poor prognosis and worse clinical outcome in a large number of tumors.

Several somatic mutations in the EGFR gene have been found to be linked with favorable response to the anti-EGFR tyrosine kinase inhibitors (TKI), Gefitinib and Erlotinib, in non–small cell lung cancer patients (40, 41). The mutations arise in four exons within the kinase domain of the receptor: point mutation of G719 in the exon 18, in-frame deletions of amino acids 746–750 in exon 19, substitution of glycine to cysteine at codon 719 (G719C) in exon 19, amino acid substitution leucine to arginine at codon 858 (L858R) and leucine to glutamate at codon 861 (L861Q) in exon 21

[111, 112]. Conversely, some mutations are able to induce resistance to TKIs such as threonine to methionine (T790M or T766M) point mutations in exon 20 (Figure 9) [53,113].

Recently two distinct therapeutic approaches have been developed for targeting EGFR: monoclonal antibodies (mAbs),which bind to the extracellular region of the receptor, inhibiting its dimerization and autophosphorylation (Cetuximab, Panitumumab) and small-molecule tyrosine kinase inhibitors (STKIs), which block receptor signaling by interfering with ATP binding to the receptor, as Gefitinib (Iressa) and Erlotinib (Tarceva), Lapatinib, Canertinib (Figure 10) [114].

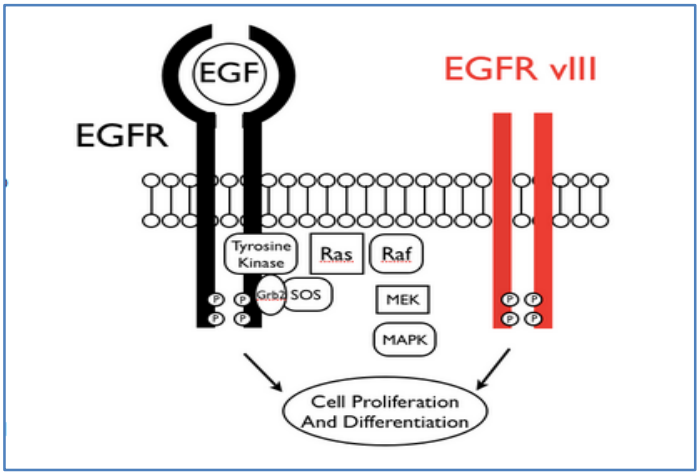


Figure 8. EGFR vIII mutant

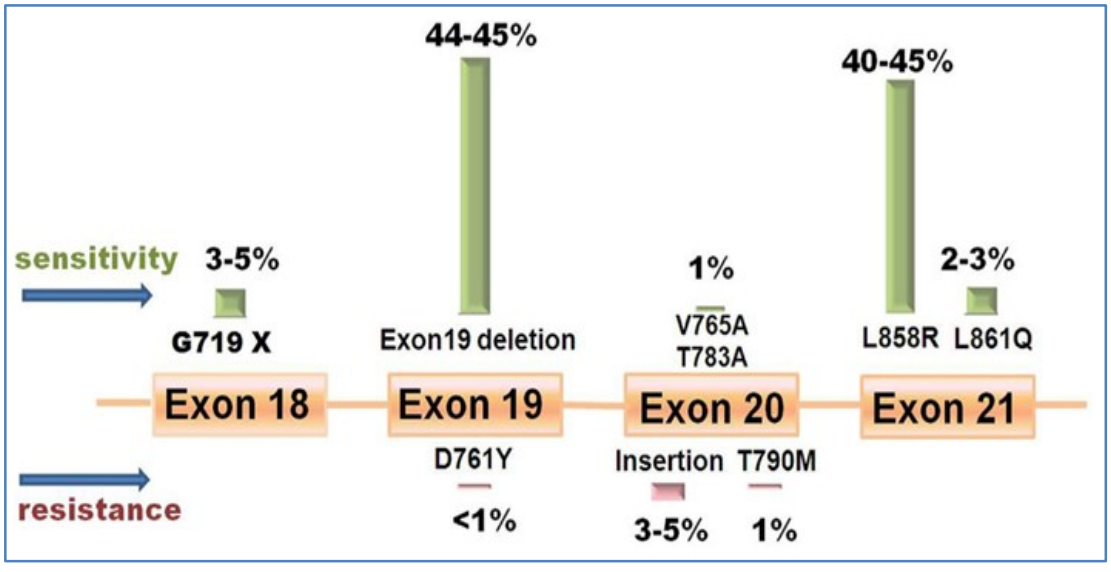


Figure 9. EGFR mutations and their frequency

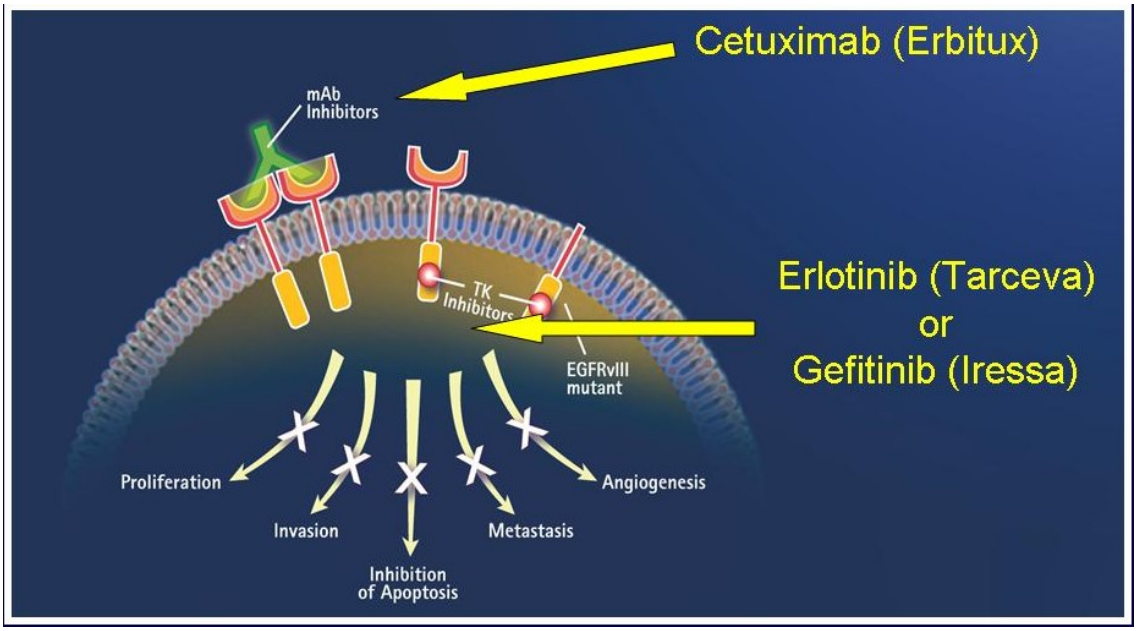


Figure 10. Targeted therapies anti-EGFR

SGLT1 (Sodium/GLucose co-Transporter 1)

The use of EGFR in CCRCC as a therapeutic target in clinical trials with tyrosine kinase inhibitors did not earn any therapeutic efficacy. The lack of responsiveness to treatment with TKI that induce blocking the activation of downstream pathways suggests the presence of a function of EGFR which is not related to its kinase activity, by which the receptor could contribute to neoplastic progression.

Evidence supported the existence of Kinase-independent function of EGFR. Several studies identified a link between glucose uptake performed by SGLT1, survival of cancer cells, and EGFR. A function of EGFR considered recently in oncology concerns its ability to promote glucose uptake into cancer cells by interacting with extracellular domain of SGLT1, through its kinase-independent mechanism, maintaining basal intracellular glucose level which avoids cell death and promotes cancer cells survival. A recent study demonstrated that the levels of SGLT1 and its transport activity vary with changes of the levels of EGFR protein.

SGLT1 is an active glucose transporter that exploits extracellular sodium concentration to transport glucose into cells independent of glucose concentration. This transporter plays an important role in maintaining glucose homeostasis both in normal cell physiology and in disease conditions.

Weihua et al. observed that EGFR can stabilize SGLT1 by protein-protein interaction which leads to prevention of SGLT1 from proteosomal degradation. The kinase-irresponsive EGFR constantly interacts with proteins regardless of the presence of EGFR ligands and activation or inactivation of its tyrosine kinase. SGLT1 is one such protein that can bind to and keep EGFR in its kinase-irresponsive status [115].

One of the key features of tumor growth is that cancer cells consume greater amounts of nutrients and energy substrates than their non-pathological counterparts, showing an altered energy metabolism. This improved energy consumption require high rate of nutrient uptake, which is realize by overexpression of plasma membrane transporters. SGLT1 induction is used by cancer cell to enhance their glucose uptake and their glycolysis, so that cells obtain sufficient energy for maintaining their expansive growth.

SGLT1 is overexpressed in different type of tumors, ovarian carcinoma, oral squamous cell carcinoma, colorectal carcinoma, pancreatic cancer and prostate cancer. In a recent study was observed that SGLT1 overexpression was significantly correlated with disease-free survival in pancreatic cancer [116, 117, 118, 119]. SGLT1 expression is also elevated in tumors with high expression of EGFR.

In another study the authors measures the expression of both EGFR and SGLT1 and tested the effect of inhibition of SGLT1 in prostate cancer tissues. These data suggest that the treatment with a SGLT1 inhibitor sensitized cancer cells to EGFR TKI; furthermore, these data demonstrate that overexpression of SGLT1 protect renal epithelial cells and intestinal epithelial cells from apoptosis [120, 121]. The deletion of the EGFR-SGLT1 interacting domain promotes the down-regulation of

SGTL1 via the proteasome machinery, suggesting that disruption of EGFR–SGLT1 interaction in EGFR-positive cancer cells may lead to down-regulation of SGLT1. It is suggested that the EGFR–SGLT1 interaction might be a novel therapeutic target for cancer treatment [120, 121].

AIMS OF THE STUDY

Pharmacological treatment by drugs targeted towards EGFR is more efficient in the treatment of those tumors that exhibit specific genetic abnormalities, such as mutations and amplification or increase in the number of copies of the EGFR gene [122], in the absence of mutations of genes that encode proteins located downstream.

The expression of EGFR protein in CCRCC has been the subject of numerous studies, performed on large series of tumors, which showed that EGFR immunoreactivity is a very common occurrence, with a overexpression range of between 50% and 90%. [2, 123-128]

Recent studies support the importance of EGFR prognostic impact in CCRCC, highlighting a positive correlation between protein overexpression and high-grade and stage of the disease, although this issue is still controversial in relation to previous experiences.

Some studies showed an association of EGFR immunoreactivity with well differentiated RCCs, or regarded strong membranous EGFR immunostaining as an indicator of good prognosis, whereas others showed an association of EGFR immunoreactivity with high tumor stage/grade and poor prognosis, or no significant associations at all [129, 130].

EGFR gene amplification and or mutations have rarely been identified in CCRCC, thus supporting the results from clinical trials that demonstrate the lack of therapeutic response of this disease to the use of molecular anti- EGFR drugs. [130-135]

Since in CCRCC genetic abnormalities responsible for EGFR protein overexpression are not apparently identifiable, EGFR overexpression should be related to alterations in the mechanisms of post-transcriptional regulation, with abnormal protein stabilization or defects in the degradation of the receptor, and consequent activation of receptor-mediated ligand. Similar mechanisms are involved in the biology of CCRCC, due to their specific genetic features, such as the loss of function of the VHL gene, resulting in a defective degradation of EGFR by poly-ubiquitination [107].

The aim of our study was to perform immunohistochemistry and molecular biology on a series of CCRCC to evaluate EGFR protein overexpression, the profile of gene expression and mutational status of the receptor, to correlate protein overexpression and gene overexpression, and to highlight the presence of any somatic mutations activating the receptor.

Furthermore, we aimed to verify the functionality of the kinase receptor by immunohistochemical evaluation of some components of the main downstream signaling pathways, such as the RAS-RAF-MAPK, PI3K-AKT, and JAK-STAT.

In addition, since it is known that EGFR possesses kinase-independent activities, which could justify its suggested role in the progression of cancer, we investigated the concurrent non-kinase role of EGFR expression by SGLT1 immunohistochemistry analysis.

MATERIALS AND METHODS

Selection of patients

A series of 34 CCRCC was selected from the archives of the Department of Histopathology of the University of Sassari. All the tumors in the sample group were reviewed and categorized according to the current classification system [21]. Three μm sections were obtained from formalin-fixed, paraffin-embedded (FFPE) specimens, for haematoxylin and eosin stains and immunohistochemical analysis.

From each selected sample 10 sections, 10 μm in thickness, were collected in sterile microcentrifuge tubes and used for the extraction of nucleic acids, used for performing genetic analysis. The healthy tissue of each sample was used as control.

Immunohistochemistry

Immunohistochemistry was performed on serial 4 μm sections from the 34 CCRCC and specific antibodies against EGFR, p-AKT, p-p44/42 MAPK, p-STAT3, SGLT1 were utilized. Sources, dilutions, antigen retrieval conditions and positive controls are summarized in Table 1. Immunohistochemistry for EGFR was performed with the EGFR pharmDx Kit (DakoCytomation, Glostrup, Denmark) according to manufacturer's instructions, as previously described [135].

Immunohistochemical results were then evaluated in a semi-quantitative manner and scored according to the intensity of immunostaining (1+, 2+, 3+) and the percentages of positively staining cells. Only cases with more than 1% of immunoreactive cells were considered as positive. Membrane and/or cytoplasmic immunoreactivity was also assessed for each positive case.

Immunoreactions for p-AKT, p-p44/42 MAPK, p-STAT3, SGLT1were obtained by incubating sections with specific primary antibodies (150 μl) for 15 minutes. Immunodetection was performed using a non-biotin, highly sensitive system (Envision peroxidase detection system, DAKO, Carpinteria, CA, USA) consequently preventing possible false-positive staining owing to endogenous

biotin present in the tissue. The slides were then incubated with DAB substrate chromogen solution for 10 minutes and counterstained with hematoxylin. Appropriate positive controls were also concurrently stained. Staining intensity and percentage of positive cells were scored for each case, with subcellular localization of immunostaining also being assessed for each positive case.

Fluorescence in situ hybridization (FISH)

The slides were deparaffinized with two washes of xylene, 15 min each, and subsequently washed twice with absolute ethanol, 10 min each and then air dried in the hood. Next, the slides were treated with 0.1mM citric acid (pH 6.0) (Zymed, CA, USA) at 95°C for 10 min, rinsed in distilled water for 3 min followed by a wash of 2XSSC (standard saline citrate) for 5 min. Digestion of the tissue was performed by applying 0.4 ml of pepsin (5 mg/ml in 0.9% NaCl, pH 1.5) (Sigma, St Louis, MO, USA) at 37°C for 30 min. The slides were rinsed with distilled water for 3 min, then washed with 2XSSC for 5 min and air dried.

Dual-color FISH was performed by using a mixture of a Spectrum green-labeled Centromeric α-satellite DNA probe (CEP7) and a Spectrum orange-labeled locus-specific DNA probe for EGFR gene. Both of the probes were from Vysis (Vysis, Downers Grove, IL, USA) and were diluted with tDenHyb2 (Insitus, Alburquerque, NM,USA) in a ratio of 1: 20.

Five µl of diluted probes were added to the slide in the reduced light condition. The slides were covered with a 22X22mm cover slip and sealed with rubber cement. Denaturation was achieved by incubating the slides at 75°C for 10 min in a humidified box and then hybridized at 37°C over night.

The coverslips were removed and the slides were washed with two washes at 45°C with 0.1XSSC/1.5M urea (20 min for each wash), followed by a wash with 2XSSC for 20 min and a wash with 2XSSC/0.1% NP-40 for 10 min at 45°C. The slides were further washed with room temperature 2XSSC for 5 min. The slides were air dried and counterstained with 10 ml DAPI (Insitus, Albuquerque, NM, USA), covered with coverslips and sealed with nail polish. The slides were

examined using an Olympus BX61 fluorescence microscope equipped with selective filters for the fluorochromes used. The images were acquired with a CCD camera and analyzed with Olympus DP-Softimage analysis software.

From each tumor section, 100 nuclei were scored for signals from LSI EGFR gene (orange) and CEP7 (green) under the fluorescence microscope with X1000 magnification and the ratio between orange and green signals was subsequently calculated; a tumor was considered amplified if the EGFR /CEP7 ratio was ≥ 2.0.

Definition of chromosomal gain was based on the Gaussian model and related to the data from the normal renal parenchyma controls, as previously described [136]. Briefly, for each slide, at least 100 nuclei were scored for signals from centromeric probes, under the fluorescence microscope with X1000 magnification, in both tumors and adjacent non-neoplastic renal parenchyma. The cut-off value for the definition of chromosome 7 gain was set at mean value plus three standard deviations (s.d.) of the percentages of nuclei with three or more signals in normal renal parenchyma. The mean percentage of nuclei with three or more signals was scored as 1,5, whereas the s.d. was assessed as 2. Therefore, the cut-off value to determine chromosomal gain in neoplastic specimens was 7.5.

Nucleic Acids Extraction

Ten consecutive sections, 10 μm in thickness, were cut under sterile conditions from representative neoplastic tissue blocks of 34 CCRCC to obtain genomic DNA, and from the same neoplastic and non-neoplastic specimens, in order to obtain total RNA. Nucleic acids were extracted with a commercially available extraction kit (Rneasy FFPE Kit and QIAamp DNA FFPE Tissue Kit, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. To obtain genomic DNA 0.2 mg of RNase A (USB Corp., Cleveland Ohio, USA) were applied directly to the silica membrane to digest contaminating RNA. We assessed the quantity and the quality of nucleic acids

spectrophotometrically (260 nm, 260/280 and 260/230 ratios, spectrum 220-320 nm) using Nanodrop ND1000 (EuroClone, Milan, Italy).

Reverse transcriptase reactions

Three µg of total RNA were reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA), complying with the manufacturer's instructions. The quality of the reverse transcription synthesis was tested by amplifying cDNA with primers of housekeeping genes (beta-actin and TBP) producing fragments of different lengths.

Quantitative Real-Time PCR (qRT-PCR)

Primers for EGFR (Hs01076078_m1, 60 bp), and 18S rRNA (Hs99999901_S1, 187 bp) human genes were chosen using Assays-on-DemandTM-Products (Applied Biosystems). Quantitative Real-Time PCR was performed using TaqMan PCR chemistry and the ABI 7900HT Sequence Detection System (Applied Biosystems). A reaction volume of 50µl containing 300 nM of primer, 200 nM of probe and 54ng of reverse-transcribed RNA (based on the initial RNA concentration) was run using the TaqMan Universal PCR Master Mix (Applied Biosystems). Cycling conditions were: 10 minutes of denaturation at 95°C, 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. RNR18 was used as the reference gene for normalizing EGFR gene expression in Real-Time PCR. Duplicate reactions were performed in each cDNA sample and the relative mRNA expression level was analyzed according to Applied Biosystem User Bulletin N°2. The calculation 2-ΔΔCt ('fold change') was chosen to represent the level of expression, with a fold change of more than 2 being considered as over-expression. Data were expressed as medians and interquartile range according to a non-normal distribution of the variables [137, 138].

Mutation analysis

EGFR gene mutation analysis was performed on exons 18 to 24, coding for the tyrosine kinase receptor domain, which are known to harbour the most frequent and significant mutations for this gene (Table 2). Gene sequencing analysis was executed as previously reported [139] .

Specifically, to detect EGFRvIII mutation, involving the deletion of exons 2 to 7, coding for the extracellular domain, a reverse transcription-PCR (RT-PCR) was developed. cDNAs were used to amplify the deletion region by primers in exon 1 (5'-GGGCTCTGGAGGAAAAGAAA-3') and exon 8 (5'-CCTCCATCTCATGCTGTCG -3'), producing a 91bp or a 892bp PCR product for EGFRvIII and EGFR wild-type, respectively.

PCR was performed in a finale volume of 20 µl, including 60ng of cDNA, 0,5 µM of each primer, 0.2 mM each of dATP, dCTP, dGTP, dTTP (Invitrogen, The Netherlands) 160mM (NH4)2SO4, 670 mMTris-HCl pH 8.8, 1.5 mM MgCl2, 5% DMSO, 2 U Super Taq (AB Analitica, Padova, Italy). PCR cycling conditions were: 5min at 94°C, 30 s at 95°C, 30 s at 60°C, 1 min at 72°C for 35 cycles, then 5min at 72°C. Cloned wild-type EGFR and EGFRvIII cDNA were used as control. Ten µl of PCR products were analyzed on a 2% agarose gel, stained with ethidium bromide.

Statistical Analysis

Statistical analysis was performed using the program Stata 11. The interaction between the clinical-pathological data and follow-up was performed by univariate analysis (p value). The comparison of the data related to the EGFR gene expression analysis in relation to histological types (neoplastic tissue to healthy tissue) were performed using non-parametric analysis by the Mann Whitney test. The limit of significance was set to $p < 0.05$.

Therefore, we assessed if there was a difference between SGLT1expression [in terms of intensity (scale 0-3), percentage of tumor cell (0-100%) and composite score, obtained by the multiplication of the intensity values * tumor cell percentage] and 4 classes of samples identified, where the first showed absence of EGFR signally pathways activation, the second activation of at

least one of the EGFR downstream pathways, the third two of the EGFR downstream pathways and the last showed activation of all EGFR pathways analyzed, using Kruskal-Wallis non-parametric statistical analysis.

RESULTS

Clinic-pathologic results

Thirty-four cases of CCRCCs, comprehensive of 4 neoplasms with sarcomatoid features, were included in the study (Figure 11). Patients' age ranged from 42 to 79 years (mean: 57.6), with predominance of males (20 cases) compared to females (14 cases). Tumor size varied between 2 and 15 cm (mean: 7 cm). Five-years follow-up data showed no evidence of disease (NED) in 17 patients, whereas 14 patients developed distant metastases, and were alive with disease (AWD); only 3 patients, all with distant metastases, died for the disease in the follow-up period. Further clinic-pathologic features are summarized on Table 3, which includes the SSIGN score.

Immunohistochemical analysis

EGFR immunostaining was reported as membranous or membranous-cytoplasmic, whereas p-AKT, and p-STAT3 showed nuclear staining, and p-p44/42 MAPK nuclear and/or cytoplasmic staining, as represented in Figure 11. EGFR expression was appreciable in 100% of the tumors, with the staining intensity ranging from 1+ to 3+, and percentages of positive cells varying from 20% to 100%. No immunoreactivity was recognizable in non-neoplastic tissues.

p-AKT expression was detected in 20 tumors (58.8%), with a 1+ to 3+ staining intensity and the range of positive cells being 10%–80%. p-p44/42 MAPK expression was identified in 18 tumors (52.9%), with a staining intensity of 1+ to 3+, and positive cell percentages between 5% and 90%.

p-STAT3 expression was recognizable in 8 tumors (23.5%) with the staining intensity ranging from 1+ to 3+, and positive cells varying from 20% to 70%. Mild to moderate, nuclear and/or nuclear-cytoplasmic immunoreactivity was also observed in endothelial cells of normal blood vessels, pericapsular adipose tissue and, focally, peritumoral renal tubules. Besides, p-AKT showed also nuclear immunostaining of renal pelvis urothelium.

Seven out of 34 tumors (20.6%) were characterized for the absence of p-AKT, p-p44/42 MAPK and p-STAT3expression, whereas 23 tumors (67.6) showed expression of at least 1 of the 3 downstream signaling pathway members. Only 4 tumors displayed concurrent immunohistochemical expression of all the 3 pathways components.

The membrane-cytoplasmic immunoreactivity for SGLT1 was detected in 31 of 34 cases (91.2%), with staining intensity between variable 1+ (15 cases), 2+ (11 cases) 3+ (5 cases); the percentage of immunoreactive neoplastic cells ranged between 10 and 90%. The positive control was represented by the epithelium of the proximal tubules, namely the luminal border, on the adjacent non-neoplastic renal parenchyma (Figure 11).

In 77.4% of SGLT1-positive CCRCCs, it showed the simultaneous activation of the EGFR kinase.

All the immunohistochemical results are summarized in Table 4.

EGFR FISH analysis

Gene amplification Ratio

The data obtained for each tumor demonstrating the absence of amplification of the EGFR gene in all cases under study; in facts, EGFR/CEP7 signals ratios ranged from 0.99 to 1.13 (average 1.03), therefore constantly lower than the value ($\geq$2) established as amplification cut-off (Figure 12).

Evaluation of chromosome 7 polysomy

This assessment was made on the basis of the analysis of the number of centromeric signals detected in non-neoplastic parenchyma adjacent to the tumor. Establishing the values of cut-off as the average percentage (M) plus three standard deviations (3SD) for the nuclei with a single signal and the nuclei with three or more signals, it is obtained a value of 7.5% for the gain of chromosome 7 (M = 1.5; SD = 2). Using the criteria for chromosome gain as described in Materials and Methods section, 11 tumors (32.3%) showed percentages of nuclei with three or more centromeric signals

below the cut-off value of 7.5%, ranging from 0 to 5% (mean: 2.9%), whereas 23 tumors (67.7%) were scored between 11 and 94% (mean: 44.9%), and considered to have gain of chromosome 7 (polysomic).

Expression Profiles Analysis

EGFR mRNA expression levels were evaluated in only 30 tumors, because expression levels were not detectable on neoplastic and non-neoplastic tissues in 4 of 34 cases.

Median values of EGFR expression level were evaluated as 3.18 (interquartile range, 1.71–5.08) in CCRCC and 0.35 (interquartile range, 0.16–0.79) in the normal tissue. Statistical significance was found between tumor and normal tissue expression levels with P-values of 0.0003. qRT-PCR results are summarized in Figure 13. Fold change evaluation for each single tumor, compared with its normal counterpart, showed overexpression levels in 12 of 30 tumors (40%) with a range from 2-fold to 17.5-fold.

EGFR Mutational Analysis

Genomic DNA sequencing of exons 18 to 24 coding for the receptor tyrosine kinase domain, which are known to harbor the most frequent and significant mutations for EGFR gene, failed to demonstrate mutations in any of the 34 cases studied.

EGFR variant III (EGFRvIII) is an oncogenic, constitutively active mutant form of EGFR. EGFRvIII is generated by in-frame genomic deletion of 801bp from exons 2 to 7 of the coding region of EGFR which produces a truncated receptor lacking a portion of extracellular ligand binding domain. All 34 CCRCCs were investigated by RT-PCR to highlight this specific deletion, but no evidence of EGFRVIII deletion was found in our neoplastic series.

Statistical Analysis

The univariate analysis of clinic-pathologic parameters with 5 years follow-up made it possible to show a strong prognostic correlation of patient survival with the following parameters: gender, grade according to Fuhrman, metastasis, tumor stage, high SSIGN score.

No equivalent significant prognostic correlation for patient survival was detected either for immunohistochemical expression of p-AKT, p-p44/42 MAPK, p-STAT3, and SGLT1, or chromosome 7 polysomy or EGFR gene expression profile. (Table 5)

The statistical analysis of the different classes of SGLT1 expression did not show any statistically significant difference ($P > 0.05$), might be due to a small number of samples in each class

DISCUSSION

Our study confirms that EGFR protein overexpression is a common molecular alteration in CCRCC.

EGFR overexpression has been previously established in CCRCC, accounting for 70% of renal cell carcinomas with a range of 47% to 93.2%; the highest frequency and positivity was recognized in CCRCC variants than other renal tumors, such as papillary carcinoma and chromophobe carcinoma [2, 140].

Our results also reveal the absence of EGFR amplification and high polysomy of chromosome 7, and are in keeping with previous experiences described in literature [2,129,131].

Sakaeda et al in 2005 showed EGFR mRNA overexpression in all series of renal cell carcinomas analyzed [141], while these results are partially confirmed in our study where the analysis of EGFR gene expression profile showed the presence of gene overexpression only in the 38.2% of CCRCC.

According to our experimental study, we were able to identify three distinct molecular subgroups in our series of CCRCC: 13 samples (38.2%) were characterized by EGFR gene and protein overexpression and activation of downstream signaling pathways; 16 samples (47%) showed EGFR protein overexpression in the absence of gene overexpression, but still with activation of downstream signaling pathways; 5 samples (14.8%) displayed only EGFR protein overexpression, whereas neither EGFR gene overexpression nor pathway activation were detected.

Our study demonstrates that CCRCC considered apparently as a molecular homogeneous tumor, is indeed more different biologically. The existence of different CCRCC subgroup can be sustained by the variability of the clinical behavior, as well as by the differences in treatment response. These molecular behaviors should be considered and analyzed for each patient, especially before proposing any treatment with biological targeted therapy.

The EGFR protein overexpression, not associated to gene overexpression but related to activation of signaling pathways, observed in some CCRCC analyzed might be related to the specific genetic alterations that characterize these tumors. Indeed, the main molecular mechanism involved in the CCRCC pathogenesis is the loss of VHL function [30, 36]. VHL gene encodes a cytoplasmic protein, that contribute at protein proteasomal degradation by complex with other ubiquitin-ligase proteins. VHL is able to reduce the EGFR protein expression by poly-ubiquitination and subsequent proteasomal degradation [107]. The EGFR stability showed in our samples might be due to the inactivation of VHL. Another mechanism responsible for EGFR degradation involves the protein c-Cbl that endorses mono-ubiquitination of the receptor and its subsequent lysosomal degradation. Mutations of c-Cbl have been identified in several kinds of malignancies, the altered protein is able to bind phosphorylated tyrosine kinase receptor, but is not able to ubiquitinate it [106]. This could be an additional mechanism to increase the EGFR availability in tumor cells.

Moreover, loss of VHL function in tumor cells results in the stabilization of HIFα, with its consequent increased transcriptional activity. Specifically, the HIFα are associated with higher expression of TGF-alpha, that is a main ligand of EGFR. Furthermore, HIFα increase the expression of caveolin 1, that under hypoxic conditions binds and promotes the dimerization and activation of EGFR in the absence of ligand [142]. Then, the stabilized protein EGFR can be activated both by an anomalous receptor dimerization in the absence of specific ligands, and by the production of TGF-alpha [40]. The consequences are the activation of downstream signaling pathways, with enhance of growth, survival, motility and metabolism of neoplastic cells.

Evidence supported the existence of Kinase-independent function of EGFR. Several studies identified a link between glucose uptake performed by SGLT1, survival of cancer cells, and EGFR [115]. A function of EGFR considered recently in oncology concerns its ability to promote glucose uptake into cancer cells by interacting with extracellular domain of SGLT1, through its kinase-

independent mechanism, maintaining basal intracellular glucose level which avoids cell death and promotes cancer cells survival [115].

Weihua et al. observed that EGFR can stabilize SGLT1 by protein-protein interaction which leads to prevention of SGLT1 from proteosomal degradation. The kinase-irresponsive EGFR constantly interacts with proteins regardless of the presence of EGFR ligands and activation or inactivation of its tyrosine kinase. SGLT1 is one such protein that can bind to and keep EGFR in its kinase-irresponsive status [115].

SGLT1 is overexpressed in different type of tumors, ovarian carcinoma, oral squamous cell carcinoma, colorectal carcinoma, pancreatic cancer and prostate cancer. A recent study demonstrated that SGLT1 overexpression was significantly correlated with disease-free survival in pancreatic cancer [116, 117, 118, 119]. SGLT1 expression is also elevated in tumors with high expression of EGFR.

The potential relation between EGFR and SGLT1 was investigated in our study showing for the first time that SGLT1 is frequently overexpressed in CCRCC EGFR-positive, whit a SGLT1 immunoreactivity detected in 31 of 34 cases (91.2%). Since the activation of downstream EGFR pathways is found in about 77% of SGLT1-positive CCRCC, it is conceivable that the functions kinase and non-kinase EGFR can be carried out independently of each other.

Moreover, the existence of 14,8% of CCRCC with only EGFR protein overexpression and absence of activation signaling pathways, but with SGLT1 overexpression might explain the neoplastic progression of CCRCC in absence of kinase activity of EGFR.

In fact, the use of EGFR in CCRCC as a therapeutic target in clinical trials with tyrosine kinase inhibitors did not earn any therapeutic efficacy. The lack of responsiveness to treatment with TKI, which induce blocking of downstream pathways, suggests the presence of a function of EGFR which is not related to its kinase activity, by which the receptor could contribute to neoplastic progression.

The deletion of the EGFR-SGLT1 interacting domain promotes the down-regulation of SGTL1 via the proteasome machinery, suggesting that disruption of EGFR–SGLT1 interaction in EGFR-positive cancer cells may lead to down-regulation of SGLT1.

According to our results the interaction between EGFR/SGLT1 might be a novel therapeutic target for cancer treatment.

Table 1. *Antibodies for immunohistochemical analyses*

Primary Antibodies	*Type*	*Dilution*	*Antigen retrieval*	*Control tissue*	*Source*
EGFR	Clone 2-18C9	Prediluted	Proteinase K (room temperature, 5 min)*	HT-29 cell line	DakoCytomation-EGFRPharmDx, Glostrup, Denmark
p-AKT (ser473)	Monoclonal	1:75	Sodium citrate buffer (pH 6, 10mM, 99°C, 20 min)	Skin	Novocastra, Dublin, OH, USA
p-p44/42 MAPK (Erk1/2) (Thr202/204)	Monoclonal	1:100	Sodium citrate buffer (pH 6, 10mM, 99°C, 10 min)	Colon carcinoma	Cell Signaling Technology, Boston, MA, USA
p-STAT3 (Tyr705)	Monoclonal	1:200	EDTA buffer (pH 8, 1M, 99°C, 15 min)	Breast carcinoma	Cell Signaling Technology, Boston, MA, USA

*available data from Kit manufacturer; min= minutes

Table 2. *Selected primers for PCR and gene sequencing*

Primers	*Sequence*	*Annealing Temperature*	*Base pair*
EGFR F exon 18	GCTTGCAAGGACTCTGGGCT	62°C	360
EGFR R exon 18	CCAAACACTCAGTGAAACAAAGAG		
EGFR F exon 19	GTGCATCGCTGGTAACATCCA	55°C	306
EGFR R exon 19	CATTTAGGATGTGGAGATGAGC		
EGFR F exon 20	GAAACTCAAGATCGCATTCATGC	60°C	379
EGFR R exon 20	GCAAACTCTTGCTATCCCAGGAG		
EGFR F exon 21	CTAACGTTCGCCAGCCATAAGTCC	57°C	370
EGFR R exon 21	GCTCACCCAGAATGTCTGGA		
EGFR F exon 22	CACTCGTAATTAGGTCCAGAG	55°C	295
EGFR R exon 22	CTCAGTACAATAGATAGACAGCAATG		
EGFR F exon 23	CAAGACTACAGAAATGTAGGTTTC	63°C	373
EGFR R exon 23	GTGATGACATTTCTCCAGGGATGC		
EGFR F exon 24	CATCACCAATGCCTTCTTTAAGC	59°C	310
EGFR R exon 24	GCTGGAGGGTTTAATAATGCGATC		

Table 3. *Clinic-pathologic features of clear cell renal cell* carcinomas

	n (%)
Pathologic Tumor Classification	
pT1a	5 (14.7)
pT1b	9 (26.5)
pT2	7 (20.6)
pT3a	7 (20.6)
pT3b	5 (14.7)
pT3c	1 (2.9)
Regional Lymph Node Involvement	
pNx	7 (20.6)
pN0	21 (61.8)
pN1	3 (8.8)
pN2	3 (8.8)
Distant Metastasis	
M0	24 (70.6)
M+	10 (29.4)
TNM Stage	
Stage I	11 (32.4)
Stage II	5 (14.7)
Stage III	6 (17.6)
Stage IV	12 (35.3)
Fuhrman Nuclear Grade	
G1	1 (2.9)
G2	8 (23.6)
G3	16 (47.0)
G4	9 (26.5)
Necrosis	
Present	17 (50.0)
Absent	17 (50.0)
SSIGN Score	
0-2	6 (17.6)
3-4	2 (5.9)
5-6	7 (20.6)
7-9	7 (20.6)
≥10	12 (35.3)

Table 4 *Immunohistochemistry results*

		EGFR			*p-AKT*			*p-p44/42 MAPK*			*p-STAT3*			*SGLT1*		
Case	*Istology*	*Intensity*	*Positive Cells (%)*	*Subcellular Localization*	*Intensity*	*Positive Cells (%)*	*Subcellular Localization*	*Intensity*	*Positive Cells (%)*	*Subcellular Localization*	*Intensity*	*Positive Cells (%)*	*Subcellular Localization*	*Intensity*	*Positive Cells (%)*	*Subcellular Localization*
1	CCRCC	2+	60	m	2+	40	n	2+	10	nc	-	-	-	1+	80	c
2	CCRCC	3+	60	mc	2+	60	n	3+	40	n	-	-	-	2+	70	mc
3	CCRCC	2+	70	mc	-	-	-	-	-	-	-	-	-	1+	70	mc
4	CCRCC	2+	70	m	-	-	-	-	-	-	-	-	-	3+	70	mc
5	CCRCC	3+	100	mc	1+	50	n	1+	10	n	-	-	-	2+	70	mc
6	CCRCC	2+	60	mc	1+	10	n	-	-	-	-	-	-	3+	80	mc
8	CCRCC	1+	40	m	3+	80	n	3+	90	n	3+	70	n	2+	80	mc
9	CCRCC	3+	90	mc	2+	70	n	3+	40	n	2+	60	n	2+	50	mc
11	CCRCC	3+	90	mc	2+	70	n	2+	40	n	2+	50	n	-	-	-
12	CCRCC	3+	90	mc	-	-	-	-	-	-	2+	30	n	3+	90	m
13	CCRCC	1+	20	m	-	-	-	-	-	-	-	-	-	2+	90	mc
14	CCRCC	2+	70	mc	2+	60	n	3+	20	nc	-	-	-	-	-	-
15	CCRCC	2+	90	mc	1+	60	n	-	-	-	2+	40	n	2+	80	mc
16	CCRCC	3+	90	mc	-	-	-	-	-	-	-	-	-	3+	80	mc
17	CCRCC	3+	80	mc	-	-	-	-	-	-	-	-	-	3+	80	mc
18	CCRCC	3+	95	m	1+	10	n	2+	60	n	-	-	-	1+	75	mc
19	CCRCC	3+	70	mc	1+	10	n	2+	20	n	-	-	-	1+	30	mc
20	CCRCC	3+	80	mc	1+	15	-	-	-	-	-	-	-	1+	25	m
21	CCRCC	3+	70	mc	1+	25	n	2+	10	n	-	-	-	2+	90	m
22	CCRCC	2+	75	m	-	-	-	1+	30	nc	-	-	-	2+	80	m
23	CCRCC	3+	80	mc	-	-	-	1+	40	n	-	-	-	1+	70	mc
24	CCRCC	3+	100	mc	1+	40	n	-	-	-	-	-	-	2+	60	mc
26	CCRCC	3+	60	m	-	-	-	3+	70	n	-	-	-	1+	70	mc
27	CCRCC-S	3+	95	mc	-	-	-	-	-	-	1+	20	n	1+	70	mc
28	CCRCC	3+	100	mc	-	-	-	1+	20	nc	-	-	-	3+	40	mc
29	CCRCC	3+	90	mc	-	-	-	1+	20	n	-	-	-	1+	60	mc
30	CCRCC	3+	90	mc	3+	80	n	3+	5	n	-	-	-	1+	90	mc
31	CCRCC-S	2+	65	mc	-	-	-	-	-	-	-	-	-	1+	30	m
32	CCRCC	1+	30	m	1+	70	n	-	-	-	3+	20	n	2+	60	m
33	CCRCC	2+	70	mc	3+	80	n	3+	20	n	-	-	-	1+	60	mc
34	CCRCC	3+	85	mc	1+	40	n	-	-	-	-	-	-	-	-	-
35	CCRCC-S	3+	90	mc	3+	80	n	3+	25	n	3+	40	n	3+	80	mc
36	CCRCC	3+	100	mc	3+	60	n	-	-	-	-	-	-	1+	5	m
37	CCRCC-S	3+	90	mc	-	-	-	-	-	-	-	-	-	3+	80	mc

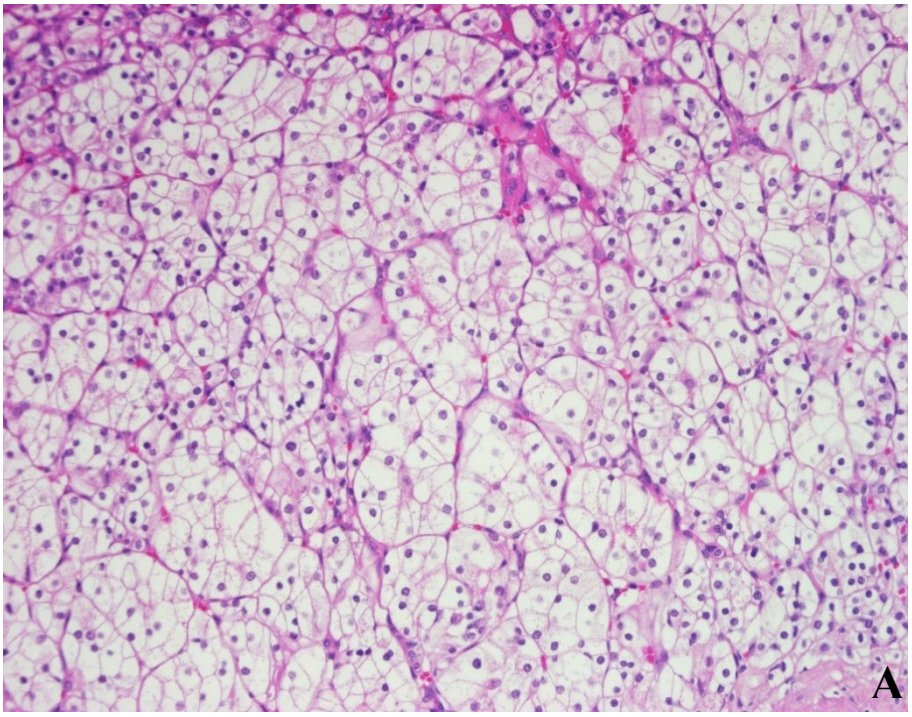
CCRCC-S: sarcomatoid clear cell renal cell carcinoma; **m**: membranous immunostaining; **c**: cytoplasmic immunostaining; **n**: nuclear immunostaining; **–** negative

Table 5. *Univariate analysis for tumor specific survival in patients with clear cell renal cell carcinoma*

Variables	*Tumors (n)*	*Tumor-specific survival (%)*	*P-value*
Age			*0.5364*
≤50	9	100	
≥50	25	0	
Sex			***0.0573***
Male	20	92.8	
Female	14	85.7	
Dimensions			*0.5495*
≤5	10	90	
≥5	24	91.7	
Grading Fuhrman			***0.0460***
G1+G2	9	100	
G3+G4	25	85.7	
Pathologic Tumor classification			*0.0684*
pT1+pT2	21	95.2	
pT3	13	84.6	
Involvement of Regional lymphnodes			*0.3339*
Nx+N0	28	92.8	
N1+N2	6	83.3	
Distant Metastasis			***0.00001***
M0	24	100	
M+	10	70	
pTNM Stage			***0.0002***
I-II	16	100	
III-IV	18	83.3	
Necrosis			*0.2982*
Present	17	94.1	
Absent	17	88.2	
SSIGN score			***0.00001***
0-2	6	100	
3-4	2	100	
5-6	7	100	
7-9	7	100	
≥10	12	75	
IHC pAKT			*0.06*
Positive	20	100	
Negative	14	78.6	
IHC p-p44/42 MAPK			*0.6113*
Positive	18	100	
Negative	16	81.2	
IHC pSTAT3			*0.7907*
Positive	8	100	
Negative	26	88.5	
IHC SGLT1			*0.7630*
Positive	31	90.3	
Negative	3	100	
Chromosome 7 polisomy			*0.2009*
Present	23	86.9	
Absent	11	100	
EGFR gene overexpression			*0.5617*
Present	13	91.3	
Absent	21	90.5	

Table 6. *CCRCC molecular subgroups identified on the basis of EGFR gene expression*

		EGFR			*p-AKT*			*p-p44/42 MAPK*			*p-STAT3*			*SGLT1*			***mRNA***
sample	*istology*	*intensity*	*Positive Cells (%)*	*Subcellular localization*	*Intensity*	*Positive Cells (%)*	*Subcellular localization*	*Intensity*	*Positive Cells (%)*	*Subcellur localization*	*Intensity*	*Positive Cells (%)*	*Subcellular localization*	*Intensity*	*Positive Cells (%)*	*Subcellular localization*	***fold change***
3	CCRCC	2+	70	mc	-	-	-	-	-	-	-	-	-	1+	7	mc	*0.54*
13	CCRCC	1+	20	m	-	-	-	-	-	-	-	-	-	2+	90	mc	*0,12*
16	CCRCC	3+	90	mc	-	-	-	-	-	-	-	-	-	3+	80	mc	*1.0*
17	CCRCC	3+	80	mc	-	-	-	-	-	-	-	-	-	3+	80	mc	*0,84*
37	CCRCC-S	3+	90	mc	-	-	-	-	-	-	-	-	-	3+	80	mc	*1,63*
1	CCRCC	2+	60	m	2+	40	n	2+	10	nc	-	-	-	1+	80	c	*0.83*
2	CCRCC	3+	60	mc	2+	60	n	3+	40	n	-	-	-	2+	70	m-c	*0,95*
5	CCRCC	3+	100	mc	1+	50	n	1+	10	n	-	-	-	2+	70	m-c	*0,55*
8	CCRCC	1+	40	m	3+	80	n	3+	90	n	3+	70	n	2+	80	m-c	*0,93*
15	CCRCC	2+	90	mc	1+	60	n	-	-	-	2+	40	n	2+	80	m-c	*1,23*
18	CCRCC	3+	95	m	1+	10	n	2+	60	n	-	-	-	1+	75	m-c	*1,50*
19	CCRCC	3+	70	mc	1+	10	n	2+	20	n	-	-	-	1+	30	m-c	*0,65*
21	CCRCC	3+	70	mc	1+	25	n	2+	10	n	-	-	-	2+	90	m	*1,40*
22	CCRCC	2+	75	m	-	-	-	1+	30	nc	-	-	-	2+	80	m	*0,44*
24	CCRCC	3+	100	mc	1+	40	n	-	-	-	-	-	-	2+	60	m-c	*0,35*
27	CCRCC-S	3+	95	mc	-	-	-	-	-	-	1+	20	n	1+	70	m-c	*0.55*
28	CCRCC	3+	100	mc	-	-	-	1+	20	nc	-	-	-	3+	40	m-c	*0,50*
30	CCRCC	3+	90	mc	3+	80	n	3+	5	n	-	-	-	1+	90	m-c	*1.2*
33	CCRCC	2+	70	mc	3+	80	n	3+	20	n	-	-	-	1+	60	m-c	*1.5*
34	CCRCC	3+	85	mc	1+	40	n	-	-	-	-	-	-	-	-	-	*0,84*
36	CCRCC	3+	100	mc	3+	60	n	-	-	-	-	-	-	1+	5	m	*0,08*
4	CCRCC	2+	70	m	-	-	-	-	-	-	-	-	-	3+	70	m-c	*5,43*
6	CCRCC	2+	60	m-c	1+	10	n	1+	<	n	-	-	-	3+	80	m-c	*4,03*
9	CCRCC	3+	90	m-c	2+	70	n	3+	40	n	2+	60	n	2+	50	m-c	*3,23*
11	CCRCC	3+	90	m-c	2+	70	n	2+	40	n	2+	50	n	-	-	-	*3,9*
12	CCRCC	3+	90	m-c	1+	<5	n	-	-	-	2+	30	n	3+	90	m	*3,82*
14	CCRCC	2+	70	m-c	2+	60	n	3+	20	n-c	-	-	-	-	-	-	*2,97*
20	CCRCC	3+	80	m-c	1+	15	n	-	-	-	-	-	-	1+	25	m	*8,29*
23	CCRCC	3+	80	m-c	1+	<5	n	1+	40	n	-	-	-	1+	70	m-c	*2,46*
26	CCRCC	3+	60	m	-	-	-	3+	70	n	-	-	-	1+	70	m-c	*2,33*
29	CCRCC	3+	90	m-c	-	-	-	2+	20	n	-	-	-	1+	60	m-c	*6,58*
31	CCRCC-S	2+	65	m-c	-	-	-	-	-	-	-	-	-	1+	30	m	*2,35*
32	CCRCC	1+	30	m	1+	70	n	-	-	-	3+	20	n	2+	60	m	*10,97*
35	CCRCC-S	3+	90	m-c	2+/3+	80	n	3+	25	n	3+	40	n	3+	80	m-c	*20,50*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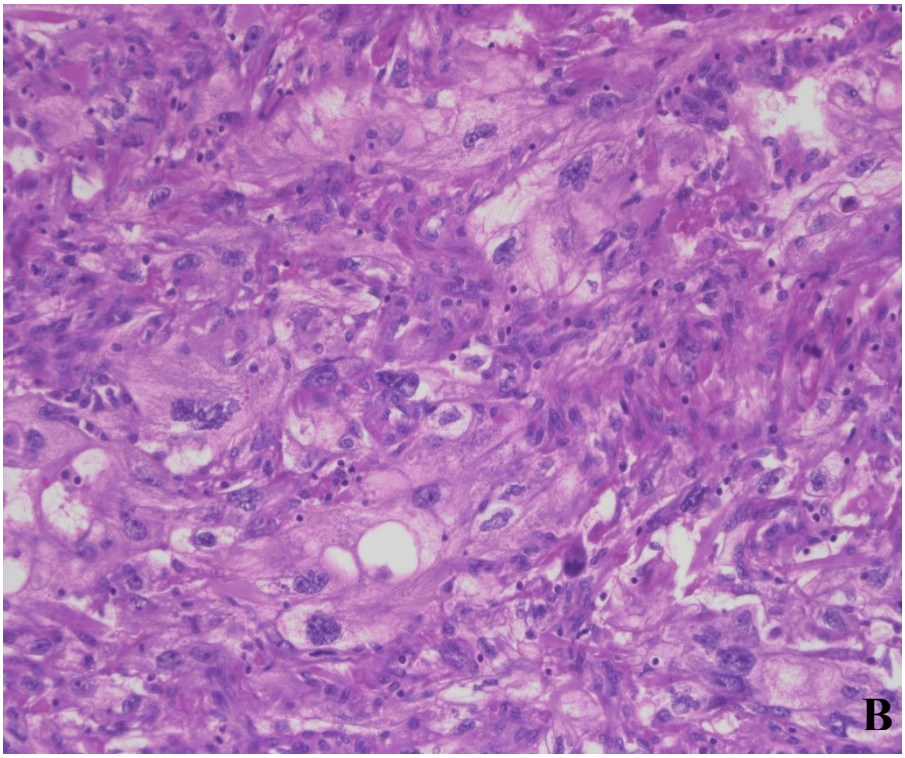
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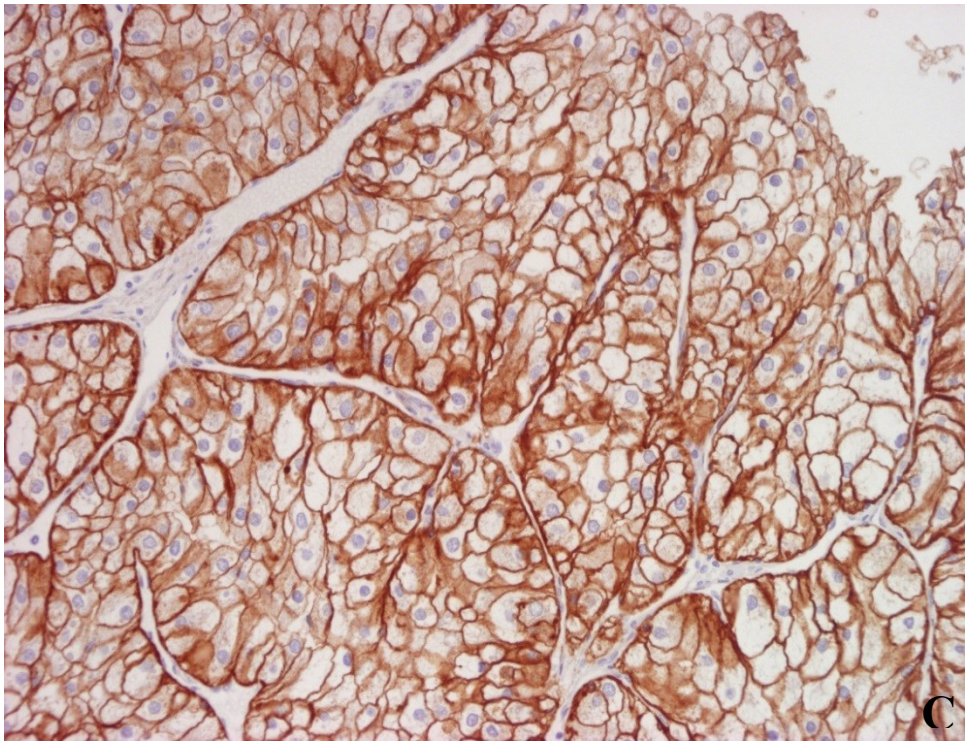
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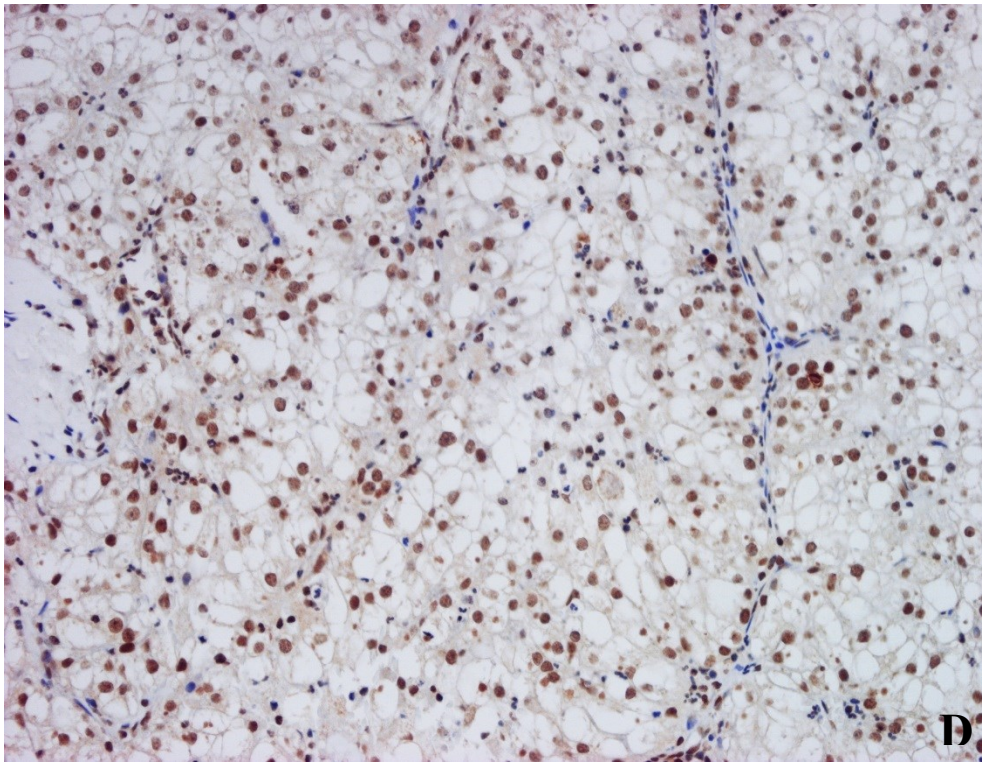
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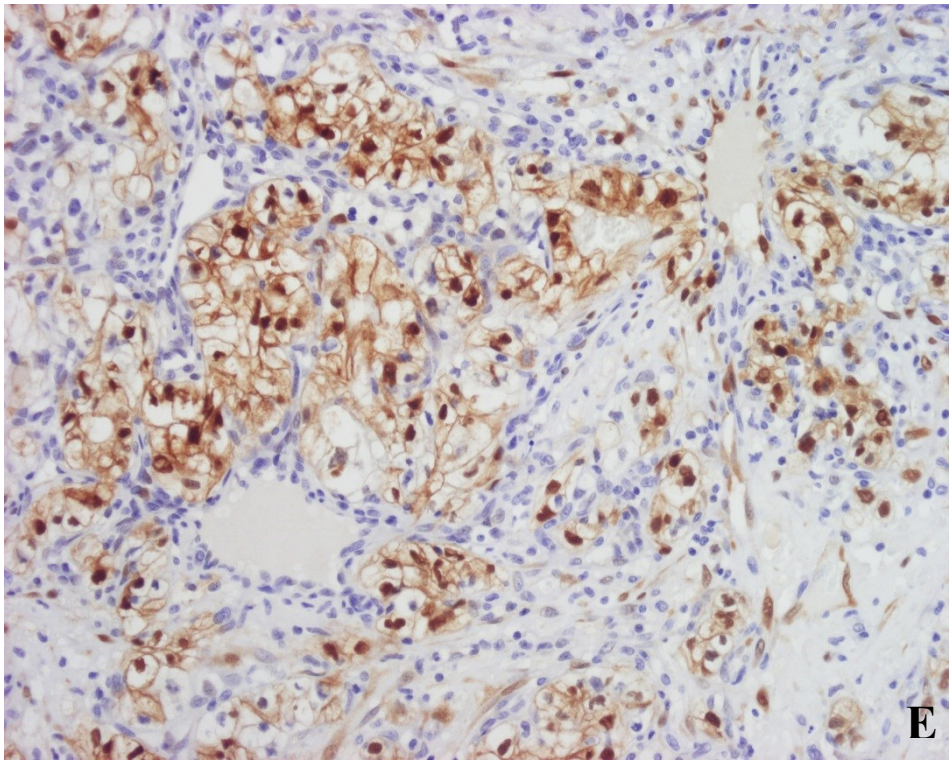
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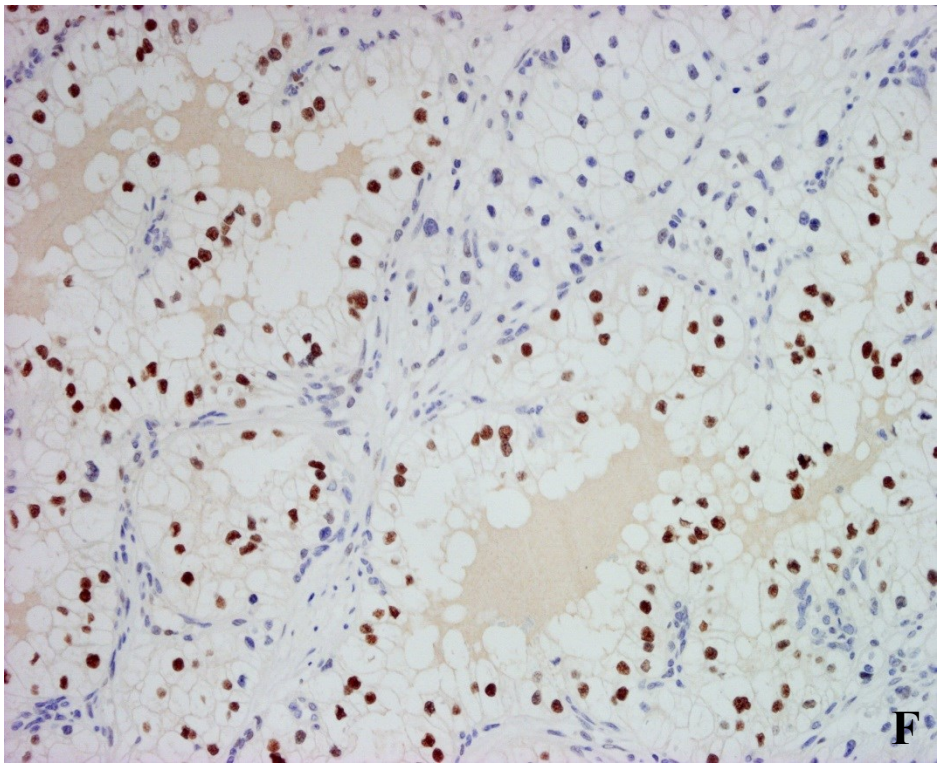
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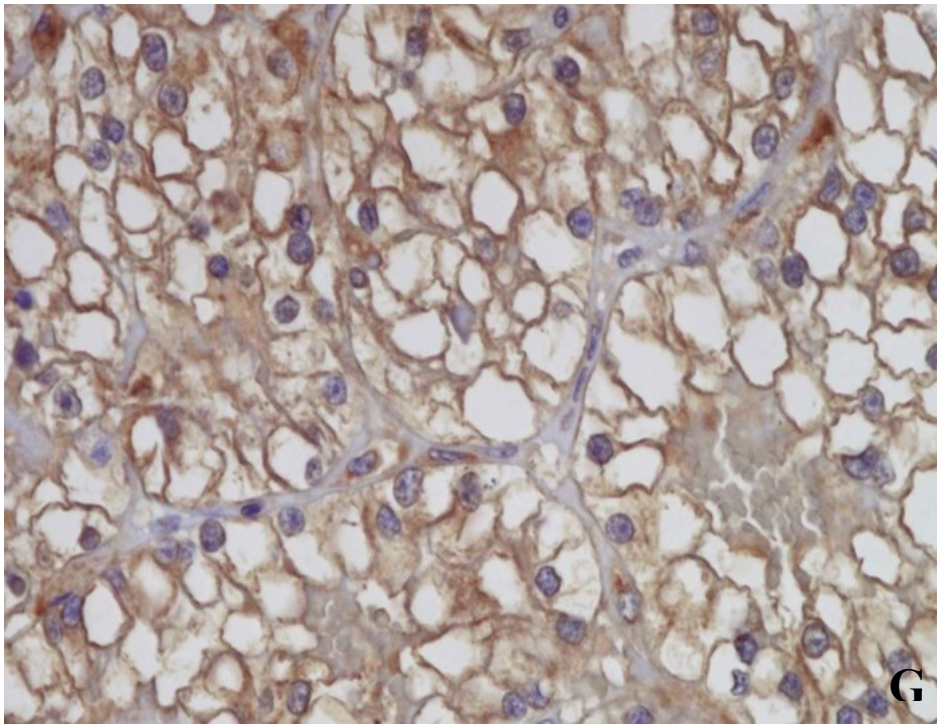


Figure 11. Morphologic and immunohistochemical features of clear cell renal cell carcinoma. a. Haematoxylin & Eosin stain illustrates the typical CCRCC features; **b.** Haematoxylin & Eosin stain illustrates the sarcomatoid CCRCC features. **c.** Immunostaining for EGFR displaying diffuse and intense membranous and cytoplasmic; **d.** Immunostaining for p-AKT showing intense and diffuse nuclear immunoreactivity; **e.** Immunostaining for p-p44/42 MAPK showing focal and intense nuclear-cytoplasmic immunoreactivity; **f.** Immunostaining for pSTAT3 displaying nuclear and moderate immunoreactivity; **g.** Immunostaining for SGLT1 displaying diffuse and intense membranous and cytoplasmic immunoreactivity.

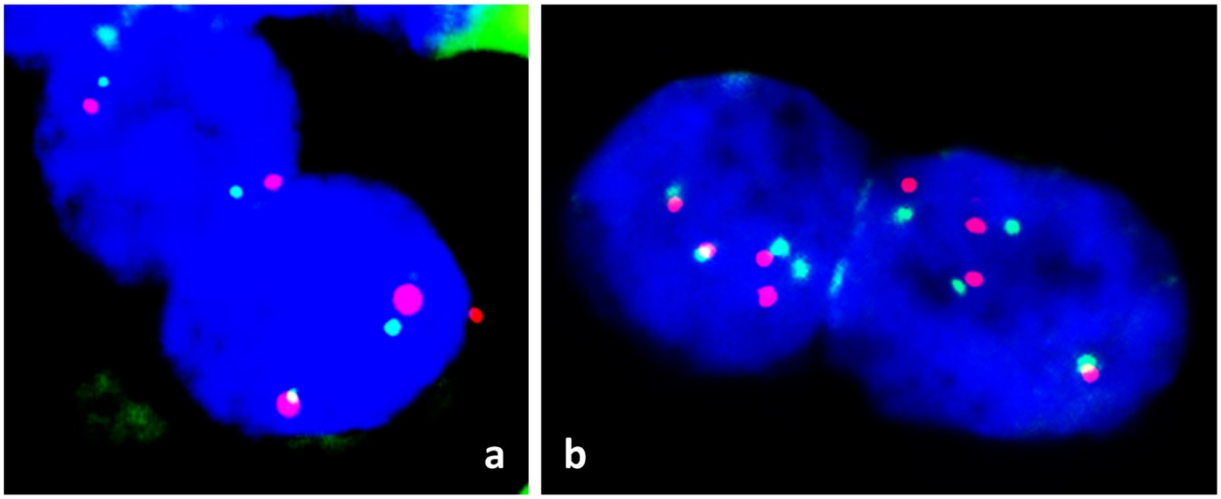
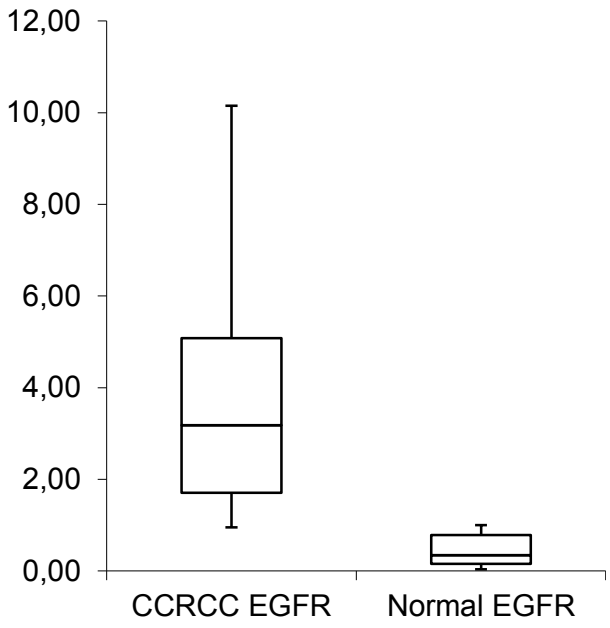


Figure 12: FISH analysis in clear cell renal cell carcinoma. a. FISH with centromeric probe for chromosome 7 showing nuclei with two green hybridization signals close two EGFR gene (red hybridization signals), consistent with absence of EGFR amplification **b.** FISH with centromeric probe for chromosome 7 showing nuclei with four green hybridization signals close four EGFR gene (red hybridization signals), indicate the presence of chr7 polysomy.



Labels	CCRCC EGFR	Normal EGFR
Min	0,95	0,04
Q_1	1,71	0,16
Median	3,18	0,35
Q_3	5,08	0,79
Max	10,97	1,00
IQR	3,38	0,63
Upper Outliers	0,00	0,00
Lower Outliers	0,00	0,00

Figure 13. Real-time polymerase chain reaction analysis for EGFR. Box and whisker plots were used to summarize the distribution of mRNA levels in clear cell renal cell carcinoma and normal tissue controls. Statistical analysis by Mann Whitney test show significant differences in mRNA levels between neoplastic and non-neoplastic tissues for EGFR gene expression, with P-value of 0.0003. The horizontal line in the box represents the 50% (median) and the upper and the lower lines of the box represent 75% and 25% quartiles, respectively

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