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MicroRNA-30a hypermethylation as prognostic marker in clear cell renal cell carcinoma

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Resumo

Introdução: O Carcinoma de Células Renais (CCR) é o cancro mais letal dos carcinomas urológicos, tendo a sua frequência vindo a aumentar nos últimos anos, devido à crescente utilização de métodos de imagem. O carcinoma de células claras (ccRCC) é o subtipo histológico mais comum e também o que apresenta pior prognóstico, comparativamente aos restantes subtipos histológicos. Os biomarcadores epigenéticos tem demonstrado uma crescente importância no âmbito do diagnóstico, prognóstico e resposta à terapêutica nos diferentes modelos tumorais. Especificamente, a metilação do DNA demonstrou desempenhar um papel fundamental na progressão tumoral, podendo também regular outros mecanismos epigenéticos, nomeadamente os micro-RNAs (miRNAs). No âmbito dos ccRCCs, o miRNA-30a demonstrou ser particularmente relevante, não só como biomarcador diagnóstico, mas também prognóstico, permitindo predizer a sobrevida livre de doença.

Objetivo: Clarificar o papel da metilação do promotor do miRNA-30a como biomarcador em ccRCCs, nomeadamente em relação ao seu valor prognóstico.

Material e Métodos: Foram utilizadas 219 amostras teciduais de ccRCCs, que incluíram ccRCCs metastizados e ccRCCs não metastizados, as quais após extração de DNA foram submetidas a modificação química por bissulfito. Seguidamente foi realizada a técnica de PCR quantitativo específico de metilação em tempo real (qMSP), sendo os níveis de metilação dos genes *miRNA-30a* e *ACTβ* obtidos e analisados estatisticamente. Para a análise do valor prognóstico, as variáveis clinico-patológicas dos respetivos doentes foram recolhidas dos ficheiros clínicos, sendo construídas as curvas de sobrevida livre de doença, utilizando o método Kaplan-Meier e o modelo de Regressão Cox.

Resultados: Não foram observadas diferenças nos níveis de metilação do miRNA-30a em doentes com ccRCCs que apresentaram metástases ao diagnóstico, comparativamente com os que não apresentaram (p=0.324), nem em doentes que recidivaram comparativamente aos doentes que não demonstraram recidiva (p=0.325). Contudo, verificou-se uma associação estatisticamente significativa entre altos níveis de metilação do miRNA-30a e estadios mais avançados (p=0.031). Relativamente ao grau de Fuhrman, não foram observadas diferenças estatísticas nos ccRCCs analisados (p=0.505). No que diz respeito à sobrevivência livre de doença, em análise univariável, níveis elevados de metilação do miRNA-30a associaram-se a um pior prognóstico (p=0.009); o mesmo foi observado para os casos com elevado grau de Fuhrman e estadio patológico (p=0.018 e p=0.017, respetivamente). Na análise multivariável, apenas níveis elevados de metilação do miRNA-

30a e estadios patológicos avançados se associaram com um maior risco de recorrência da doença (p=0.030 e p=0.044, respetivamente). Assim, a metilação do miRNA-30a e o estadio patológico demonstraram ser variáveis independentes de prognóstico para sobrevida livre de doença nos ccRCCs.

Discussão e Conclusões: Os níveis de metilação do miRNA-30a e o estadio patológico podem ser usados como variáveis de prognóstico em ccRCCs, sendo preditores independentes da sobrevida livre de doença, o que poderá ser uma ferramenta útil na prática clínica de forma a identificar doentes com alto risco de recorrência. Contudo, ao diagnóstico, os níveis de metilação do miRNA-30a não discriminam doentes com metástases dos sem metástases.

Palavras-chave: microRNA-30a; Metilação do Promotor; Carcinoma de Células Claras Renal; Prognostico; Biomarcador; Epigenética

Abstract

Introduction: Renal Cell Carcinomas (RCCs) are the most lethal among urological cancer. Due to the widespread use of imaging, the frequency of incidental detection of RCCs has significantly increased. The most common RCCs are clear-cell RCC (ccRCC), which have the worse prognosis when compared to other histological subtypes. Epigenetic-based biomarkers may assist in diagnosis, prognostic stratification and prediction of response to targeted therapy in cancer. Specially, DNA methylation has been demonstrated to play a key role in cancer progression and could regulate other epigenetic mechanisms, such as micro-RNAs (miRNAs). In the context of ccRCCs, miRNA-30a have been demonstrated to have a relevant role, not only as diagnostic biomarker but also as prognostic biomarker, predicting disease-free survival.

Aim: To clarify the role of miRNA-30a promoter methylation in ccRCCs and its prognostic value.

Materials and Methods: DNA was extracted from a total of 219 tissue samples of ccRCCs, including metastatic ccRCCs and non-metastatic ccRCCs. Then, DNA was modified by bisulfite reaction and miRNA-30a methylation levels were determined by quantitative real-time Methylation Specific PCR (qMSP) and analysed by statistical analysis. The prognostic significance of available clinical variables was assessed by constructing disease-free survival, using Kaplan-Meier method and Cox-regression model.

Results: MiRNA-30a methylation levels did not differ significantly between ccRCCs patients that presented metastases or relapsed at diagnosis compared with those without metastatic disease or recurrence ($p = 0.324$ and $p=0.325$, respectively). In our series, a significant association was found between miRNA-30a methylation levels and stage ($p=0.031$), as higher methylation levels of miRNA-30a were significantly found in advanced stages (III&IV); however, no statistical significance was found for Fuhrman grade ($p=0.505$). Regarding disease-free survival, in a univariable analysis, higher miRNA-30a methylation levels associated with a worse prognosis ($p = 0.009$); the same was observed for cases with high Fuhrman grade and stage ($p = 0.018$ and $p = 0.017$, respectively). In the multivariable analysis, only high miRNA-30a methylation levels and advanced stages associated with a shorter time to relapse ($p = 0.030$ and $p = 0.044$, respectively). Thus, miRNA-30a methylation and stage are independent prognostic factors for disease-free survival.

Discussion and Conclusions: MiRNA30a promoter's methylation levels and stage are independent predictors of disease-free survival and may identify ccRCCs patients who are at risk of recurrence. However, no differences were apparent concerning miRNA-30a methylation levels in patients without metastasis from those harbouring metastases at the diagnosis.

Keywords: microRNA-30a; Promoter methylation; clear cell Renal Cell Carcinoma; Prognostic; Biomarker; Epigenetics

List of Abbreviations

AJCC: American Joint Committee on Cancer

ccRCC: clear cell Renal Cell Carcinoma

ccRCCm: metastatic ccRCC

chRCC: chromophobe Renal Cell Carcinoma

CT: Computed Tomography

DFS*:* Disease-free survival

DLL4*: Delta-like 4*

DNMT: DNA methyltransferase

FFPE: formalin-fixed paraffin-embedded

H3K4me3: trimethylation of lysine 4 of histone H3

H3K27Ac: acetylation of lysine 27 of histone H3

HIF: Hypoxia Inducible Factor

IFN-α*: interferon alpha*

IL-2*: Interleukin-2*

MBD: methyl-CpG-binding domain

miRNAs: microRNAs

MRI: Magnetic Resonance Imaging

MSP: Methylation Specific PCR

mTOR: Mammalian Target of Rapamycin

NaOH: Sodium Hydroxide

PDGF*: Platelet-derived Growth Factor*

pRCC: papillary Renal Cell Carcinoma

PTM: post-translational modifications

qMSP: quantitative real-time MSP

RCC: Renal Cell Carcinoma

SDS: Sodium dodecyl sulfate

TSS: Transcription Start Site

US: Ultrasound

VEGF*: Vascular Endothelial Growth Factor*

VHL*: von Hippel-Lindau Tumor Supressor*

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Introduction

Kidney Cancer

Worldwide, kidney cancer accounts for nearly 2.4% of all adult malignancies, being the 13th most common malignancy[1]. The incidence varies substantially worldwide, with the highest rates being generally registered in Europe and North America and the lowest in Asia and South America. According to Globocan, in 2012, 337 860 new cases and 143 369 deaths were attributable to kidney cancer worldwide, for both sexes[1]. In the same year, Portugal registered 665 new cases of kidney cancer in males and 339 in females, and 240 deaths in men and 128 in women were accounted to kidney cancer[1].

Renal Cell Carcinoma

Renal Cell Carcinoma (RCC) is the most common carcinoma in kidney and it is derived from renal tubular epithelial cells. It encompasses a heterogeneous group of cancers with different histological subtypes, based on morphological and genetic characteristics[2]. Three major subtypes are recognized, clear-cell RCC (ccRCC), papillary RCC (pRCC) and chromophobe RCC. ccRCC accounts approximately 70% to 80% of all RCCs being the most common histological subtype, while the pRCC represents 10-15% of RCC, whereas chRCC accounts for 5-7%[3].

Clear-Cell Renal Cell Carcinoma

ccRCC is the predominant histologic subtype of RCC and it is found in more than 80% of patients who develop metastases[2]. ccRCC arise from the epithelial cells of the proximal tubules[3]. Their causes are not fully understood yet, but specific lifestyle factors have been recognized as important etiologic factors for this neoplasia.

Two of most important risk factors are gender and age, as ccRCCs are more common in men, with a male to female predominance of 2:1, and they occur primarily in elderly patients, typically within the sixth and seventh decades of life[4, 5]. Most of ccRCC are sporadic, although familiar forms have been reported, as such as von Hippel-Lindau (VHL) syndrome. Many environmental factors have been investigated as possible contributing factors in which cigarette smoking was identified with a strong association[6].

Concerning macroscopic features, ccRCCs are typically well-circumscribed and lobulated, with a yellow cut surface due to the high lipid content of the tumor cells. Microscopically, ccRCC has a regular network of small thin-walled blood vessels presenting varied architecture, with solid,

alveolar and acinar patterns. CcRCCs primarily metastasize via the vena cava, to the lung, although lymphatic dissemination may also occur[3].

Concerning to genetic changes, chromosome 3 alterations and *von Hippel-Lindau Tumor Suppressor (VHL)* mutations are common in ccRCC; indeed, *VHL* gene mutation or inactivation by hypermethylation was found in the majority (>70%) of ccRCC sporadic cases[3, 7]. *VHL* inactivation is thought to be pivotal for ccRCC carcinogenesis. VHL protein targets Hypoxia Inducible Factors (HIFs) to ubiquitin-mediated degradation. In the absence of *VHL* protein, HIFs accumulate and pro-survival and pro-angiogenic factors start to be transcribed, as *Vascular Endothelial Growth Factors (VEGF)* and *Platelet-derived Growth Factor (PDGF*)[8-10].

ccRCCs are most commonly detected as incidentalomas because of the widespread use of imaging methods as part of abdominal pain or other unrelated diseases. Consequently, many renal tumors are diagnosed at early and less aggressive stages, in which treatment is usually more effective. The classic triad of flank pain, hematuria and palpable abdominal mass are nowadays rarely found. Other symptoms such as fever, weight loss, anemia and varicocele are also less frequent. Because of the retroperitoneal location of the kidney, many renal masses remain asymptomatic and no palpable until advanced stages[2, 11].

The current approach for renal masses detection and characterization is based on imaging techniques as Computed Tomography (CT), Magnetic Resonance Imaging (MRI) or Ultrasound (US)[11]. Imaging techniques are useful to classify renal masses as solid or cystic and also to classify solid masses as benign or malignant, as the presence of enhancement is predictable of malignant lesions; CT scan is the most important exam for delineating the nature of renal masses, while MRI and US are used when CT scans are indeterminate or there are contraindications for CT use[2].

Staging is based on the American Joint Committee on Cancer (AJCC) Tumor, Node and Metastasis (TNM) staging system (Supplementary A

Table 1) and for RCC there are four stages, varying between stage I and stage IV, according to aggressiveness[2].

Concerning treatment, localised disease or advanced disease must be approached differently. Currently, depending on tumor size and location, renal function, comorbidities and performance status, different strategies for localised ccRCC management are available, including nephrectomy, thermal ablative therapies or active surveillance[4]. Notwithstanding advances in the understanding of the genetics and biology of RCC, surgery remains the standard for curative treatment[11]. Because, metastatic ccRCC (ccRCCm) is commonly refractory to chemotherapy and surgery has a limited role, different approaches are required. Immunotherapy is one of the options to offer to patients with ccRCCm, as interferon alpha (IFN-α) and Interleukin-2 (IL-2) are the most

used immune modulators in clinical practice[12]. Recent advances in molecular biology led to the development of novel agents for ccRCCm treatment that block important pathways (angiogenesis, cell cycle regulation) in renal carcinogenesis such as *VEGF*, *PDGF* and Mammalian Target of Rapamycin (mTOR) pathways[2, 8, 13]. Current challenges include the development of more selective drugs targeting these and others undiscovered pathways important for ccRCC carcinogenesis, that are actually being developed[8, 12, 13].

Comparing to the other histological subtypes, ccRCC has a worse prognosis, even after stratification for stage and grade[14]. The main prognostic factors used in clinical practice, include TNM staging system and pathologic stage as they reflect tumor biology[2]. In fact, the higher tumor stage, associates with worse prognosis, as in stage I >90% of patients is alive in 5-year surveillance, whilst patients in stage IV, which has a 5-year surveillance of 10[11]. Concerning to histological factors, Fuhrman nuclear grade system is also an independent prognostic factor with 5-year survival rates of 64%, 34%, 31% and 10% for grades 1 to 4, respectively[9]. Clinical factors such as the Eastern Cooperative Oncology Group (ECOG) performance status are also important prognostic factors[2].

Epigenetics

Epigenetics was firstly introduced in 1939 by Waddington, who defined it as “the causal interactions between genes and their products, which bring the phenotype into being”; currently epigenetics refers to heritable changes in gene expressions that are not due to any alteration in the DNA sequence[15]. It is now acknowledged that epigenetics is implicated in a wide variety of biological processes, not only during embryonic development but also during differentiation. A disruption in the normal balance of epigenetic marks can result in a deregulation of various signalling pathways, and consequently can lead to disease states such as cancer[16].

Currently, three main epigenetic mechanisms are recognized: DNA methylation, post-translational modifications of histone proteins and chromatin remodelling and non-coding RNAs. These three mechanisms, which interact with each other, are dynamic and they work together in order to regulate gene expression[17].

DNA Methylation

DNA methylation is the most extensively studied epigenetic modification in mammals. It consists in the addition of a methyl group, by DNA methyltransferases (DNMTs), at the 5’ position of a cytosine ring within CpG dinucleotides, mainly found in CpG islands[18]. CpG islands are characterized by a CG content of 50% at least and a ratio of observed/expected CpG dinucleotides

of at least 0.6 [17]. Moreover, CpG islands are present in about 60% of human gene promoters, which makes DNA methylation an important regulatory mechanism of gene transcription[19, 20].

In normal cells, DNA methylation is implicated in maintenance of genomic imprinting, transcriptional regulation, developmental processes and genome integrity. In fact, this alteration occurs predominantly in repetitive genomic regions to maintain genomic integrity[20]. Concerning gene expression, CpG islands DNA methylation is generally associated with gene repression and with chromatin repressive states. This transcription inhibition occurs directly by blocking the binding of specific transcription factors and indirectly by recruitment of methyl-CpG-binding domain (MBD) proteins, which in turn recruit histone modifying and chromatin-remodelling complexes to methylated sites which ultimately mediate gene repression[17, 20].

Histone Post-Translational Modifications and Chromatin Remodelling

The nucleosome is the basic unit of chromatin and it is composed of an octamer of histone proteins around which 146bp of DNA are wrapped. Histone are small basic proteins containing a globular domain, which directly interacts with DNA, and a flexible charged N terminal, also known as the histone tail, which protrudes from the nucleosome and can be altered by different post-translational modifications, such as methylation or acetylation[20]. The octamer that compose nucleosome consists of two subunits of each of the following core histone proteins: H2A, H2B, H3 and H4[18].

Chromatin structure is controlled by two main classes of protein complexes: those that remodel nucleosomes in an ATP-dependent manner and those that covalently modify histone tails and involve the recruitment of chromatin modulators[21]. Both determine chromatin status, which can be a more condensed pattern, called heterochromatin, which is associated to inactive genes, or a more uncondensed configuration, euchromatin, associated to actively transcribed genes[18].

Chromatin remodelling complexes are multi-subunit complexes that use ATP hydrolysis to alter the interaction of DNA and nucleosome and therefore alter the conformation of chromatin; these complexes include for example chromatin helicase DNA-binding proteins (CHDs) family[22]. The modulation of chromatin conformation though covalent post-translational modifications (PTMs) of histones is another fundamental mechanism of gene transcription regulation and it includes methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, or deamination[23]. Histone modifications are thought to alter the electrostatic charge of the histones, resulting in a changing in their binding of DNA, which in turn results in different status of euchromatin or heterochromatin and consequently in altered transcriptionally activity. The most studied and well characterized are histone acetylation and methylation, which are established by several enzymes with variable residue-specificity. Whereas histone acetylation occurs at lysine

residues and is associated with a more open chromatin conformation and thereby leads to gene activation, histone methylation is not so clearly predictable as the consequences on DNA transcription depends on the residue affected as well as the degree of methylation (mono, di or trimethylation)[23]. Histone modification levels are predictive of gene expression and recent studies have shown that generally, actively transcribed genes are characterized by high levels of trimethylation of lysine 4 of histone H3 (H3K4me3) and acetylation of lysine 27 of histone H3 (H3K27Ac)[17, 20].

MicroRNAs

MicroRNAs (miRNAs) are a class of non-coding RNAs. Non-coding RNAs are RNAs that do not encode for proteins and are regulators of gene expression being described as crucial in several cellular pathways[24]. In fact, they have been implicated in different molecular events in eukaryotic cells, including chromosome dynamics, splicing, RNA editing, inhibition of translation and mRNA destruction. Non-coding RNAs comprise several different classes, according to their length and their function [24]. MiRNAs are endogenous single-stranded non-coding RNAs, with 18 to 25 nucleotides in length, which are synthesized in the nucleus and then exported to the cytoplasm[25]. Their effect on mRNAs regulation depends on the level of complementarity between miRNA and its target mRNA sequence[24]. Initially, miRNA have been reported as negative regulators of mRNA expression, however recent data have indicate an opposite effect, partly explained by binding sites of miRNAs in target mRNA[26]. Each miRNA may regulate multiple mRNAs and, conversely, each mRNA may be targeted by multiple miRNAs. In fact, it is estimated that 30 to 70% of human genes are regulated by miRNAs, in a temporal and tissue specific manner. Hence, altered miRNAs can dramatically affect a variety of cellular processes, namely proliferation, cell death, differentiation and development[20, 24]. Although the mechanism underlying miRNA deregulation in cancer is not fully understood, it is known that DNA methylation is involved in the regulation of miRNA expression[17]. New technologies have enabled comprehensive analysis of the epigenome and as a consequence, the list of miRNAs silenced by methylation in cancer is growing rapidly. Indeed, many families of miRNAs have already been described as being silenced by methylation, including the miR-124, miR-34, miR-9, miR-200 and miR-205 families[27].

Epigenetic Interaction mechanisms: DNA methylation and miRNAs

Epigenetic mechanisms can interact between each other, reflecting its integrated nature. In fact, miRNAs can modulate epigenetic regulatory mechanisms by targeting enzymes responsible for DNA methylation, such as DNMT3A and DNMT3B, and histone modifications, as EZH2[20]. Specifically, miR-29 family members are downregulated in several cancers and they are predicted

to target DNMT1, DNMT3A and DNMT3B. Also, miR-34b has shown to target DNMT1 in prostate cancer cell lines, as well as it is also epigenetically silenced by DNA methylation [28]. Furthermore in ccRCC, DNA promoter methylation levels were inversely correlated with miR-21, miR-10b and miR-30a expression [29].

Epigenetics and ccRCC

As in other cancer subtypes, different epigenetic mechanisms have been identified during RCC tumorigenesis and progression. In fact, its identification has potential use in molecular diagnosis, prognosis and possible therapeutic approaches[16].

Concerning DNA methylation, 70% of sporadic ccRCC cases are characterised by alterations on *VHL* gene, in which 15% of these alterations are caused by aberrant promoter methylation, highlighting the importance of DNA methylation in ccRCC pathogenesis[7]. Different studies comparing DNA methylation profiles between ccRCCs and normal renal tissues has also found numerous genes frequently methylation in tumors, including *CDH1, APAF1, COL1A1, DKK2, DKK3, SFRP1, SFRP4, SFRP5, WIF, PCDH17* and *TCF21*[30]. Another CpG methylation study which used 38 ccRCCs and 9 matched normal kidney tissues identified 55 genes methylated in tumor samples but not in normal kidney tissues; these genes included *OVOL1, DLEC1, BMP4, SST, TMPRSS2, TM6SF1*, *SLC34A2* and *COL1A2*[31]. Besides its role in ccRCC tumorigenesis, DNA aberrant methylation is also a feasible prognostic tool, reflecting tumor behaviour of ccRCC. In fact, methylation of *FAM150A*, *GRM6, ZNF540, ZFP42, ZNF154, RIMS4, PCDHAC1, KHDRBS2, ASCI2, KCNQ1, PRAC, WNT3A, TRH*, *FAM78A, ZNF671, SLC13A5* and *NKX6-2* genes were associated with a more aggressive phenotype and a poor outcome[30]. Moreover *APAF1*, *DAPK1*[32], *PTEN*[33] and *JUP*[34] genes were associated to a worse prognosis, even in a multivariate analysis.

Regarding histone modifications recent data has associated this epigenetic alteration and hypoxic cellular response. Indeed, hypoxia was reported to be associated to loss of H3K9Ac, increase in H3K9me2 and H3K9me3, typically associated with gene repression, and also with increased in H3K14Ac, H3K4me2, H3K4me3 and H3K7me3, associated to gene activation[35, 36]. Several histone marks have also been associated to a poor prognosis including H3K4me2, H3K18ac and H3K9me2[37]. Conversely, EZH2 upregulation was associated to a favourable prognosis[38].

Deregulation of miRNA expression was also found in ccRCCs, with diagnostic and prognostic value. miR-16, miR18a and miR21 are upregulated in ccRCC and miR-141 and miR-200b are described to be downregulated. Nowadays, several pathways were identified as being deregulated in ccRCC by miRNAs, which includes metabolism, focal adhesion, cell cycle regulation and apoptosis[39]. Particularly, the miR-200b family is involved in in epithelial to mesenchymal transition

and its downregulation might contribute to tumor invasion and metastasis[39, 40]. For biomarker detection, a study demonstrated that a combination of miR-141 and miR-155 was capable of distinguish ccRCCs from normal kidney tissue. Concerning to prognosis, miRNA expression could be associated to survival or some prognostic factors as overexpression of miR-32, miR-210, miR-21 and miR-18a are correlated to poor survival[41, 42]. Also, higher miR-210 levels were found in tumors with higher Fuhrman grade[43]. Furthermore, lower miR-106b levels were associated with metastatic disease[44] and high expression levels of miR-210 was correlated to with lymph node metastasis[43].

Micro-RNA 30a Family

MiRNA-30a belongs to the miRNA-30 family that is codified on chromosome 6q13. This family of miRNAs has been implicated in a widespread of biological processes, including cellular differentiation and development[45]. Indeed, miRNA-30a has been reported to promote chondrogenic differentiation via downregulation the expression of Delta-like 4 (DLL4). Moreover, miRNA-30 family was found to be regulated by Wnt/ β-catenin pathway[45].

In human cancer, miRNA-30a has been implicated in cellular proliferation, invasion, metastasis and autophagy. Specifically in RCC, miRNA-30a was shown to have a critical role in autophagy, as it inhibits the autophagy through downregulation of beclin-1, interfering with the effectiveness of sorafenib, a multi-kinase inhibitor used in RCC treatment[46]. Moreover, miRNA-30a was significantly downregulated in RCC tissues compared to normal adjacent kidney tissues and in RCC cell lines[46].

In a study using microarrays, miRNA-30a was demonstrated to be under-expressed in metastatic ccRCCs when compared to non-metastatic ccRCCs. Furthermore, miRNA-30a lower expression were correlated with a worse survival[47] and was found to be an independent predictor of ccRCC hematogeneous metastases by the univariate analysis[48].

Recently, Wang and colleagues[49] not only reported that miRNA-30a was downregulated in ccRCCs tissues compared to adjacent normal tissues, as well as in 5 different RCC cell lines compared to a normal kidney cell line, but these authors also found that miRNA-30a low expression levels was associated to a high TNM stage and an advanced pathological grade in ccRCC patients. In survival analysis, a shorter overall survival time was displayed by these patients compared to RCC patients with high miRNA-30a expression [49].

Aims of the Study

Because miRNA-30a expression levels were found to be deregulated in ccRCCs and miRNA expression may be regulated by DNA promoter methylation, the aims of this study are:

I. **Assess whether miRNA-30a aberrant promoter methylation might be a metastasis biomarker in ccRCCs at the time of diagnosis.**
   1. Evaluate the methylation levels of miRNA-30a in metastatic ccRCCs and non-metastatic ccRCCs;
   2. Correlate methylation levels of miRNA-30a with metastasis and recurrence status;
   3. Correlate methylation levels of miRNA-30a with clinicopathological data (stage and Fuhrman grade).

II. **Evaluate the prognostic value of miRNA-30a promoter methylation in ccRCCs.**
   4. Correlate methylation levels of miRNA-30a with disease free survival.

Materials and Methods

Patients and Sample Collection

In the present study, a total of 219 were prospectively collected from patients consecutively diagnosed and submitted to nephrectomy at the Portuguese Institute of Oncology – Porto, Portugal. All specimens were immediately frozen after surgical procedure and stored at -80ºC for further analysis. The presence of tumor cells was confirmed by staining with Haematoxylin and Eosin (H&E), before and after the frozen sections were cut, ensuring sampling of more than 70% of malignant cells. Relevant clinical data, namely recurrence dates, TNM stage and Fuhrman grade was also collected. This study was approved by the Institutional Review Board (CES-IPOFG-EPE 518/10) of Portuguese Institute of Oncology - Porto, Portugal.

DNA Extraction

DNA was extracted by phenol-chloroform method. Briefly, the digestion of tissues was the first step, by adding tissue digestion buffer (composed by SE solution - 75mM Sodium Chloride [EMD-Millipore and 25 mM EDTA] and 10% of Sodium Dodecyl Sulphate (SDS) [Sigma Aldrich®]) and proteinase K (Sigma-Aldrich®), followed by an overnight incubation in a water-bath at 55ºC until total digestion was achieved. In some cases, the incubation went for 2 to 3 days and proteinase K was added twice a day, until digestion was achieved. Then, phenol/chloroform solution at pH8 (Sigma Aldrich®) was added in Phase Lock Gel Light tubes of 2mL (5 Prime, Deutschland, Germany) and the upper aqueous phase was collected to a new tube. Then, DNA precipitation was performed by adding 100% cold ethanol, 7.5 M ammonium acetate (Sigma-Aldrich®) and 5 mg/mL glycogen (Applied Biosystems®), which were mixed and incubated overnight at -20^{o}C. Finally, the samples were washed in successive washes with 70% ethanol solution and the pellets were air dried and eluted in 30 µL of sterile distilled water (B. Braun, Melsungen, Germany). DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies) and stored at - 20ºC until used.

Bisulfite Modification

After DNA extraction, samples were submitted to a sodium bisulite treatment, which allow the identification of methylated and unmethylated cytosines. The basic principle of sodium bisulfite modification of DNA is that all unmethylated cytosines are deaminated, sulphonated and then converted to thymines, whereas methylated cytosines remain unaltered in the presence of Sodium Hydroxide (NaOH) and sodium bisulfite[50]). Consequently, the sequence of treated DNA will differ

depending on whether the DNA is originally methylated or not, since unmethylated cytosines are converted to uracil residues and methylated cytosine remain as cytosines. The sodium bisulfite modification was performed using EZ DNA Methylation-Gold™ kit (Zymo Research, Orange, CA, USA), according manufacturer's guidelines with 1 µg of DNA in a total volume of 20 µL in sterile distilled water used. The denaturation of samples was performed in Veriti® Thermal Cycler, using manufacturer's conditions. CpGenome™ Universal Methylated DNA (EMD-Millipore, Temecula, CA, USA) and CpGenome™ Universal Unmethylated DNA (EMD-Millipore) were also modified to be used as positive and negative controls of the experiment. Finally, samples were eluted in 60µL of sterile distilled water and stored at -80^{o}C, while controls were eluted 30 µL and stored at -20ºC.

CpG islands were searched in 2000 base pairs upstream of Transcription Start Site (TSS) of *miRNA30a* and a pair of Methylation Specific PCR (MSP) primers were designed by Methyl Primer Express® v 1.0. The sequence of MSP primers for miRNA-30a are: Forward 5'*TAGTCGAGGATGTTTATAGTCG*3' and Reverse 5'*AACTTCAATACTTTACAAAATCG*3'. For ACTβ the sequence are: Forward 5'*ACCAATAAAACCTACTCCTCCCTTAA*3' and Reverse 5'*TGGTGATGGAGGAGGTTTAGTAAGT*3'.

Quantitative Real-Time Methylation Specific PCR

To assess methylation levels, quantitative real-time MSP (qMSP) was performed in all samples using KAPA SYBR FAST qPCR Kit Master Mix®. The modified DNA was used as template and samples were submitted to reactions with the target gene, miR-30a, and the reference gene, ACTβ.

Reactions were carried out in 384-well plates using LightCycler 480 II (Roche, Germany). Briefly, for miRNA-30a, it was added 1 µL of modified DNA, 5 µL of 2X KAPA SYBR FAST qPCR Master Mix, 0.3 µL of primer mix and 3.7 µL sterile distilled water in order to total 10 µL of reaction volume, per each well; for ACTβ, the volumes for modified DNA and 2X KAPA SYBR FAST qPCR Master Mix were the same as miRNA-30a, except to primer mix and sterile distilled water, which were 0.4 µL and 5.0 µL, per each well.

The PCR program consisted of a period of 3 minutes at 95°C for enzyme activation followed by 45 cycles with 3 seconds at 95°C (for DNA denaturation) and 30 seconds at 60°C (for annealing, extension and data acquisition).

All samples were run in triplicates and in each plate one negative template control was run. Modified CpGenome™ Universal Methylated DNA® was used to create five serial dilutions by a 5x dilution factor. These serial dilutions were run in each plate and were used to generate a standard

curve thus allowing absolute quantification as well as ascertaining PCR efficiency. All plates had an efficiency between 90-100%.

Methylation levels were calculated as a ratio between the target gene mean quantity and ACTβ mean quantity:

$$Methylation\ level = \frac{miRNA - 30a\ mean\ quantity}{ACT\beta\ mean\ quantity}$$

Statystical Analysis

Non-parametric tests were performed to determine statistical significance in all the comparisons made. Kruskal-Wallis test was used in comparisons between 3 or more groups, whereas Mann-Whitney U test was used for comparisons between two groups. Qui-Square Test was used to seek for differences in the miRNA-30a promoter methylation according to the stage categorization and Fuhrman grade. Survival function was performed to evaluate correlation between methylation levels and disease specific survival for the 219 ccRCCs patients included in this study. The prognostic significance of available clinical variables (metastatic ccRCC or non-metastatic ccRCC, stage, Fuhrman grade and tumor size) was assessed by constructing disease-free survival curves using the Kaplan-Meier method with log-rank test (univariable test). The methylation levels of miRNA-30a were classified as low or high based on the cut-off value of 25th percentile. A Cox-regression model using ENTER method comprising the different variables (multivariable test) was also constructed.

Statistical analysis was performed using SPSS software for Windows, version 23.0 (IBM-SPSS Inc.), and graphs were built using GraphPad Prism 6.0 software for Windows (GraphPad Software Inc.). Statistical significance was set at $p<0.05$.

Results

The relevant clinical and pathological characteristics of patients included in this study (n=219) are depicted in Table 2. MiR-30a methylation levels did not significantly differ between metastasized patients (n=6) from those patients that did not harbour metastasis at the diagnosis (p=0.324) (Figure 1). Furthermore, no significant differences were found between patients who relapsed (n=38) and patients who did not relapsed (p=0.325) (Figure 2).

The distribution of methylation levels of miRNA-30a by stage and Fuhrman grade are illustrated in Figure 3 and Figure 4, respectively. In our series, a significant association was found between the 25th percentile miRNA-30a methylation levels and stage (p=0.031), as higher methylation levels of miRNA-30a (>P25) were significantly found in advanced stages (III&IV) (Figure 5). No statistically significance was found for Fuhrman grade (p=0.505) (Figure 6).

For Disease-free survival (DFS) analysis (n=211) 8 patients were excluded, 6 because presented metastasis at the diagnosis and other 2 because never presented remission of the disease. The median follow-up of ccRCC patients was 61 months (range: 1-195 months). Higher miR-30a methylation levels significantly associated with poor prognosis (shorter time to recurrence, p=0.009) (Figure 7).

In univariable analysis (Kaplan-Meier), higher stage (≥III) and Fuhrman grade (≥3) associated with shorter survival (p=0.017 and p=0.018, respectively) (Figure 8 and Figure 9). Tumor size did not disclose any prognostic value within the available follow-up time (p=0.084, data not shown).

In multivariable analysis (Cox regression), miRNA-30a methylation levels and stage predicted shorter disease-free survival. Indeed, a higher recurrence risk was depicted by patients with higher stage and higher miR-30a methylation levels (Table 3).

Discussion

Over the last decade, due to the widespread of imaging techniques, the frequency of incidental detection of RCCs has significantly increased. ccRCCs are the most common RCCs, which have the worse prognosis when compared to other histological subtypes, namely in what concerns to the frequency of distant metastasis, which is about 20%[14]. Indeed, distant metastases are the main cause of the high mortality rate of RCCs, making these carcinomas the most lethal among the common urologic cancers[2]. Thus, there is an urgent need of biomarkers capable of accurately discriminate tumours that will metastasize from those that will not, especially among those of small dimension (mostly pT1). Epigenetic-based biomarkers may assist in diagnosis, prognosis stratification and prediction of response to targeted therapy[16]. In fact, DNA methylation has been demonstrated to play an important role in cancer progression by regulating protein coding and Non-coding genes as miRNAs[51]. In addition, miRNA-30a expression levels demonstrated to be deregulated in ccRCC and were reported to convey prognostic significance[47-49]. In this context we assessed whether miRNA-30a methylation levels might be a metastisation biomarker in ccRCCs. To achieve that goal, miRNA-30a methylation levels were evaluated in 219 ccRCCs (metastatic ccRCCs and non-metastatic ccRCC). Furthermore, the potential significance of miRNA-30a methylation levels as prognostic biomarker on disease free survival was also evaluated.

Herein, miRNA-30a aberrant methylation levels at the diagnosis did not significantly differ in patients with metastasis from those patients who did not harbour metastasis. Moreover the same occurs for patients who present recurrence during follow up time from those who did not relapse. In literature, the studies comparing ccRCCs and miRNA-30a were about expression levels and none was conducted using methylation levels of miRNA-30a. In a study led by Heinzelmann and colleagues it was demonstrated that miRNA-30a expression levels distinguishes between metastatic and non-metastatic ccRCC[47], which was not possible to prove in our study. However it is important to mention the results published were about a microarray experiment, which used only eighteen samples (10 from non-metastatic ccRCCs and 8 from metastatic-ccRCCs) and when these results were tried to be validate in a large series, the statistically significance could not be demonstrated[47]. Most of the studies showed a downregulation of miRNA-30a in tumor samples when compared to non-tumoral kidney tissues. In fact, this was demonstrated by Huang and colleagues which showed a miRNA-30a downregulation in tumor tissues and even a further decreased expression levels in hematogenous metastatic ccRCCs, demonstrating that miRNA-30a expression levels could be an independent prognostic factor in predicting haematogenic metastization[48]. Wang and colleagues also showed in ccRCCs, a miRNA-30a downregulation in

tumors samples when compared to matched normal tissues. Moreover, these results were paralleled by renal cell lines studies. The same authors were able to demonstrate that higher miRNA-30a expression levels associated with enhanced proliferation in cancer cell lines, whereas respective knockdown augmented colony formation and promoted anti-apoptosis, thus suggesting an oncogenic activity for this miRNA in RCC[49]. Moreover, in a recent study, miRNA-30a overexpression in 769-P cells prevented cellular proliferation and invasion; and in mice significantly decreased tumor volume[52].

Aberrant promoter methylation, globally inversely correlates with miRNA-30a expression levels[29], we expected that methylation levels significantly differed between metastasized patients from those patients without metastasis, nonetheless we were not able to confirm that in our series. This might be due to the fact that other mechanisms have been also associated with miRNA-30a expression levels regulation, including other molecules involved in autophagy pathways[46]. However, we demonstrated that higher methylation levels are associated with advanced stages, which could emphasize the role of promoter methylation on stratifying patients according to biological behaviour of ccRCCs.

Conversely, in disease-free survival analysis, higher miRNA-30a methylation levels significantly associated with poor prognosis. Indeed, our results on methylation levels are in line with the reported decreased miRNA-30a expression levels in hematogenous metastatic ccRCC. In this study by Huang et al, miRNA-30a expression levels were found to be independent predictors of ccRCC hematogenous metastasis, along with tumor size. Indeed, the risk of hematogenous metastasis in miRNA-30a low-expression levels tumors was 9 fold higher than the tumors expressing high miRNA-30a levels[48]. In accordance with our results, other authors recently reported that miRNA-30a downregulation associated with TNM and advanced pathological grade in ccRCCs patients. Indeed, Kaplan-Meier survival analysis demonstrated that patients with lower miRNA-30a expression had a shorter overall survival time compared ones with high expression levels[49].

Considering that all the above mentioned studies evaluated miRNA-30a expression levels in tissue samples and that methylation assessment displays several advantages, including higher DNA stability, reduced amount of clinical material and methodological celerity comparing with RNA expression assays, methylation analysis would easily applicable to daily clinical practice for non-invasive testing using either urine or liquid biopsies[53, 54]. Indeed, it was already showed that miRNA-30a DNA promoter methylation inversely correlated with respective expression in ccRCCs[29], which further supports our initial hypothesis. Thus, although the global sample size is relatively high (n=219), the number of metastatic ccRCCs is low, with 6 patients, representing 2.7% of our series,

thus impacting in the lack of significant association between miRNA-30a methylation levels and the risk of developing metastasis in ccRCCs.

Conclusions and Future Perspectives

In this study, although miRNA-30a methylation levels did not significantly differ between metastatic ccRCC and non-metastatic ccRCC, higher miRNA-30a methylation levels significantly associated with a poor prognosis in these patients. Indeed, stage and higher miRNA-30a methylation levels predicted disease-free survival, thus, being useful in predicting the patients at higher risk of recurrence and/or metastization.

As future perspectives, it would be interesting to determine the expression levels of miRNA-30a in order to conclude whether there was an inversely association with methylation levels in our series. Moreover, *in vitro* studies could also be relevant to determine the expression and methylation levels of miRNA-30a in ccRCC cell lines and to understand the pathways involved on its regulation such as proliferation, invasion and apoptosis assays. In what concerns to survival we intend to assess miRNA-30a methylation levels in a ccRCC series with longer follow-up, to evaluate its clinical applicability in predicting metastasis development, and therefore stablish an individualized management. Furthermore, it will be also interesting to evaluate the expression and methylation levels of other miRNA-30 members, since members such as miRNA-30c were suggested to play a potential role in ccRCC tumorigenesis and metastasis development.[47]

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Supplementary

Supplementary A

Table 1 - TNM Staging System for Renal Cell Carcinoma proposed by the American Joint Committee on Cancer in 2009. Stage I tumors are <7cm in greatest diameter and confined to the kidney; stage II tumors are >7cm and confined to the kidney; Stage III extend though the renal capsule but are confined to Gerota's fascia (IIIa) or involve a single hilar lymph node (N1); stage IV disease includes tumors that have invaded adjacent organs (excluding adrenal glands) or involve multiple lymph nodes or distant metastases.

T: Primary Tumor			
Tx	Primary tumor cannot be assessed		
T0	No evidence of primary tumor		
T1	Tumor ≤7.0 cm and confined to the kidney		
T1a	Tumor ≤4.0 cm and confined to the kidney		
T1b	Tumor >4.0 cm and ≤7.0 cm and confined to the kidney		
T2	Tumor >7.0cm and confined to the kidney		
T2a	Tumor >7.0 cm and ≤10.0 cm and confined to the kidney		
T2b	Tumor >10.0cm and confined to the kidney		
T3	Tumor extends into major veins or perinephric tissues but not into the ipsilateral adrenal gland and not beyond the Gerota fascia		
T3a	Tumor grossly extends in the vena cava below the diaphragm		
T3c	Tumor grossly extends into the vena cava above the diaphragm or invades the wall of the vena cava		
T4	Tumor invades beyond the Gerota fascia (including contiguous extension into the ipsilateral adrenal gland)		
N: Regional Lymph Nodes			
NX	Regional lymph nodes cannot be assessed		
N0	No regional lymph nodes metastasis		
N1	Metastasis in regional lymph node(s)		
M: Distant Metastases			
MX	Distant metastasis cannot be assessed		
M0	No distant metastasis		
M1	Distant metastasis present		
Stage Grouping			
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1 or T2	N1	M0
	T3	Any N	M0
Stage IV	T4	Any N	M0
	Any T	Any N	M1

Supplementary B

Table 2 - Clinical and Pathological data of patients included in the present study.

	ccRCC
Number of Patients, n	219
Median age, years (range)	64 (29-86)
Gender, n (%)	
Male	139 (63.5)
Female	80 (36.5)
ccRCC, n (%)	
ccRCCm	6 (2.7)
Non-ccRCCm	213 (97.3)
Stage, n (%)	
I	113 (51.6)
II	33 (15.1)
III	53 (24.2)
IV	7 (3.2)
N.A.	13 (5.9)
Fuhrman Grade, n (%)	
1	6 (2.7)
2	88 (40.2)
3	100 (45.7)
4	24 (11.0)
N.A.	1 (0.5)

N.A.: not available

Supplementary C

Table 3 - Prognostic factors in Disease-free Survival obtained by Cox regression multivariable analysis using Enter method. The high and low levels of miRNA-30a methylation levels were categorized using 25th percentile value as cut-off.

Prognostic Factor	Hazard Ratio (HR)	95% CI for HR	Cox regression p value
miR-30a methylation (high *vs* low levels)	3.707	1.136 – 12.101	0.030
Stage (high *vs* low stage)	1.936	1.019 – 3.680	0.044

Supplementary D

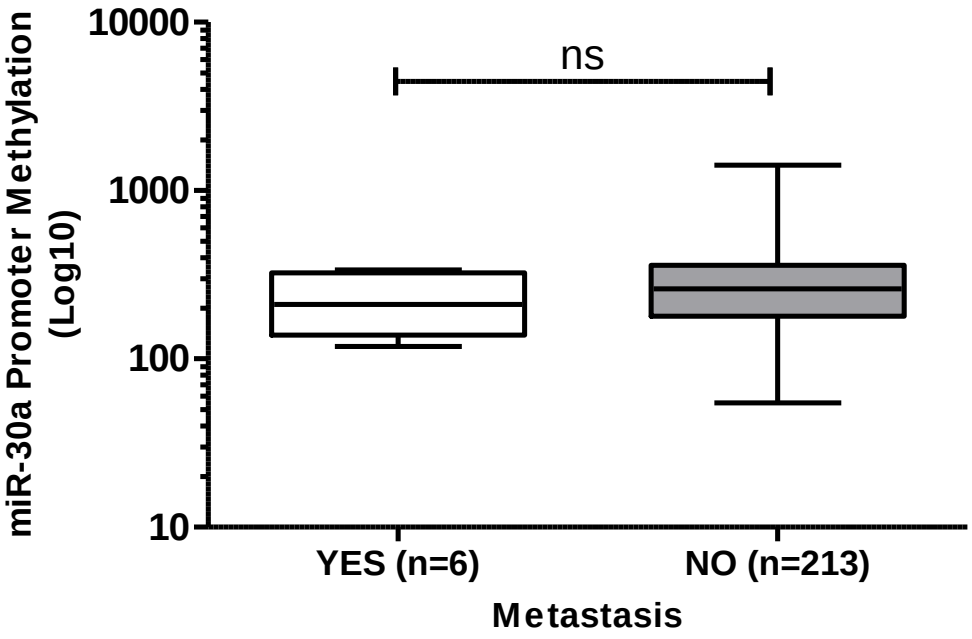

Figure 1 – Distribution of miRNA-30a methylation levels in clear cell renal cell carcinomas (ccRCC) according with metastasis status.

Supplementary E

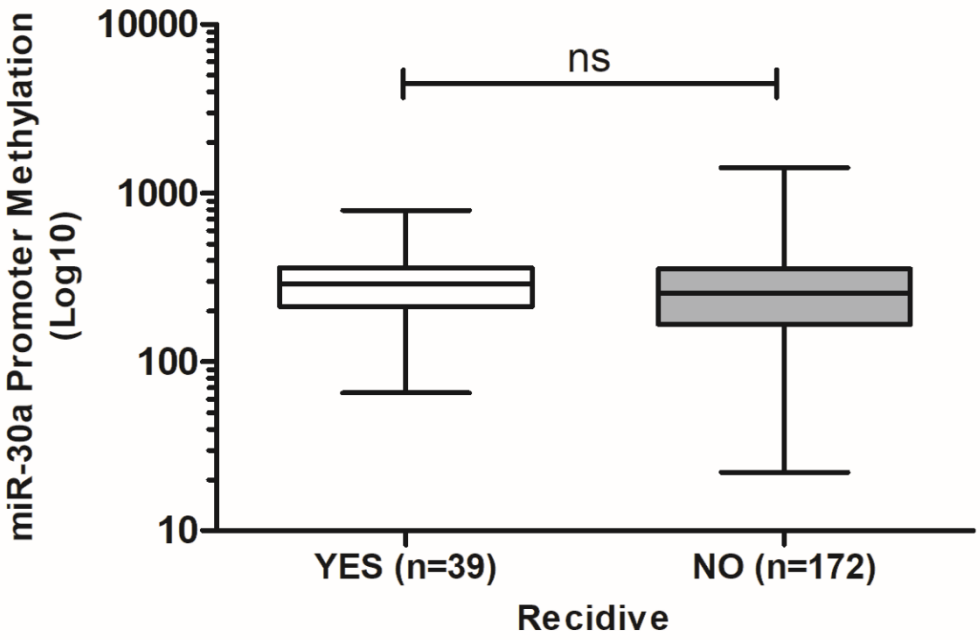

Figure 2 - Distribution of miRNA-30a methylation levels in clear cell renal cell carcinomas (ccRCC) according with recurrence status.

Supplementary F

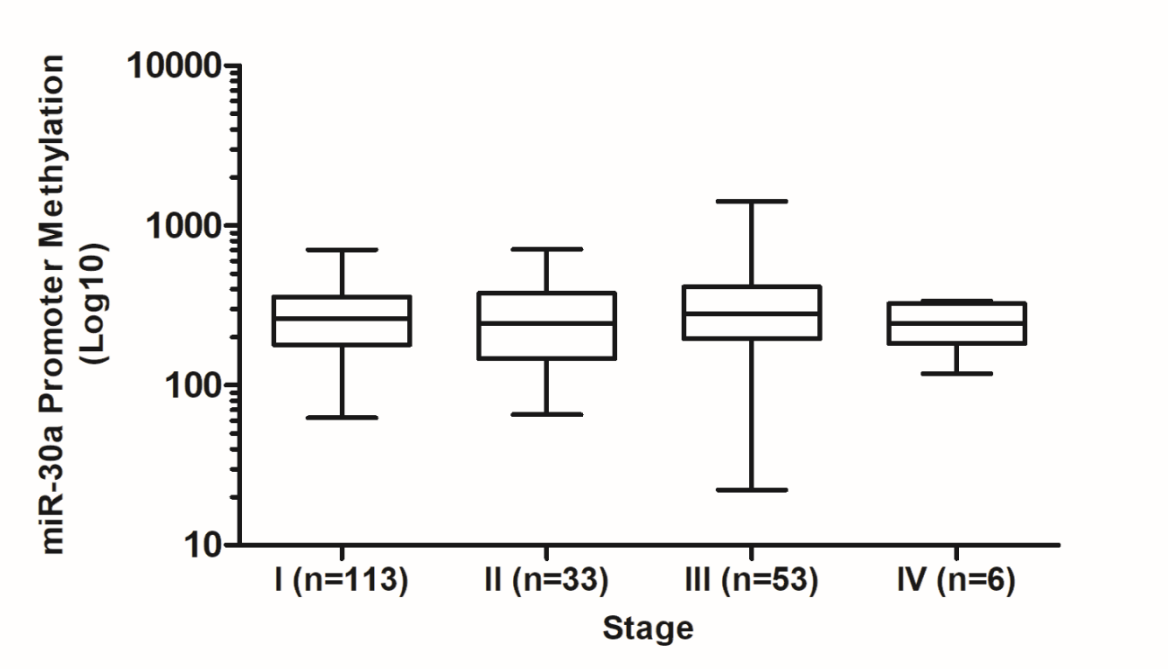

Figure 3 – Distribution of miRNA-30a´s methylation levels by stage.

Supplementary G

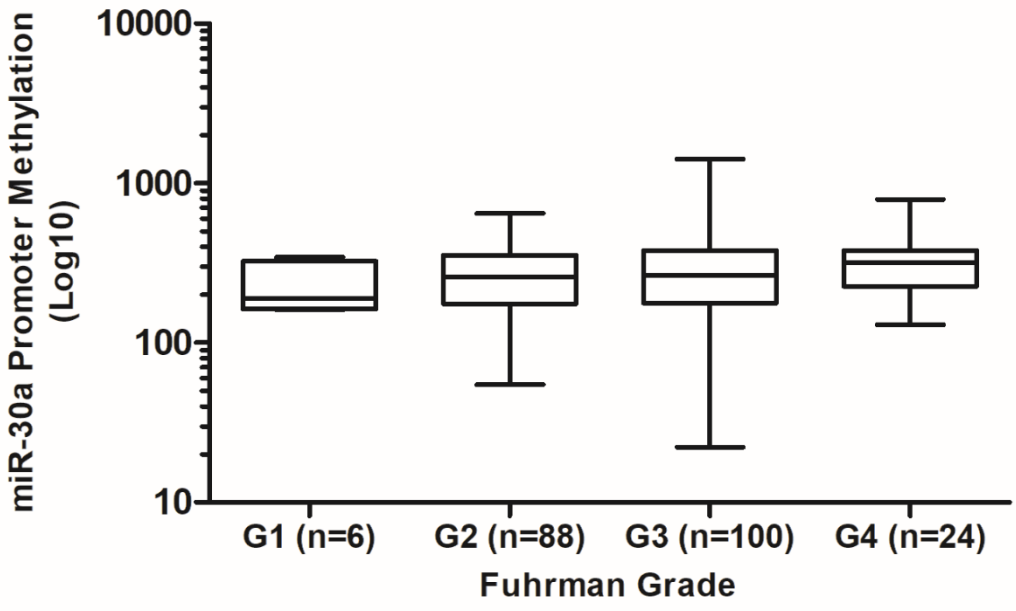

Figure 4 - Distribution of miRNA-30a's methylation levels by Fuhrman grade.

Supplementary H

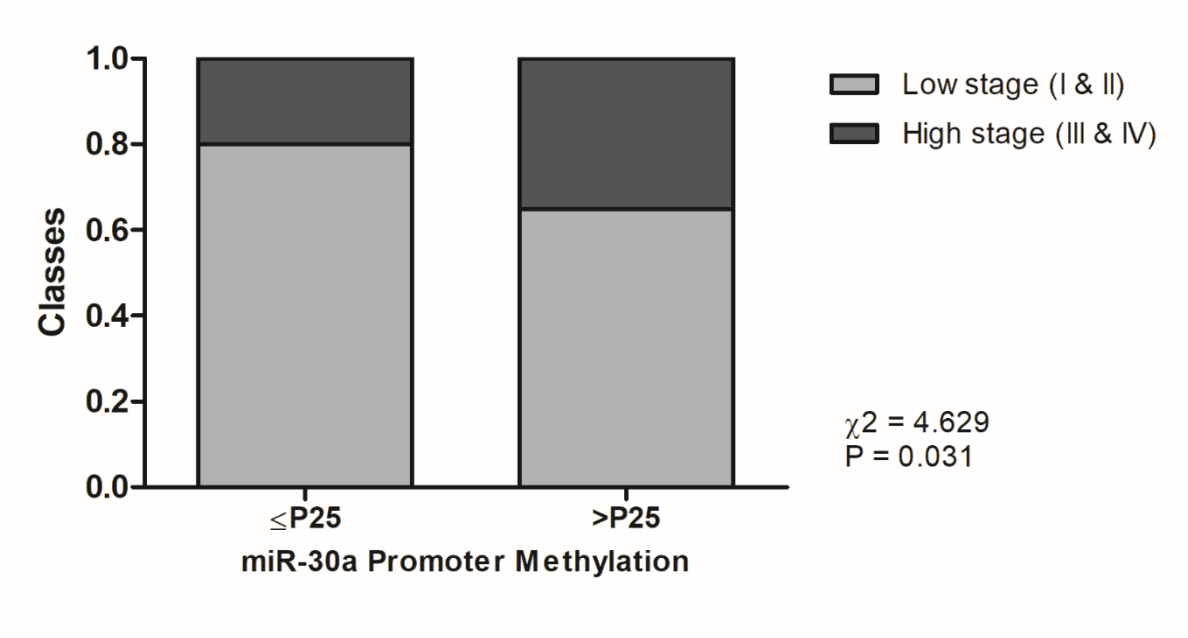

Figure 5 – Distribution of 25th percentile of miRNA-30a methylation levels according to low and high stage. The Stage I and II were categorized as Low Stage, and Stage III and IV were categorised as High stage.

Supplementary I

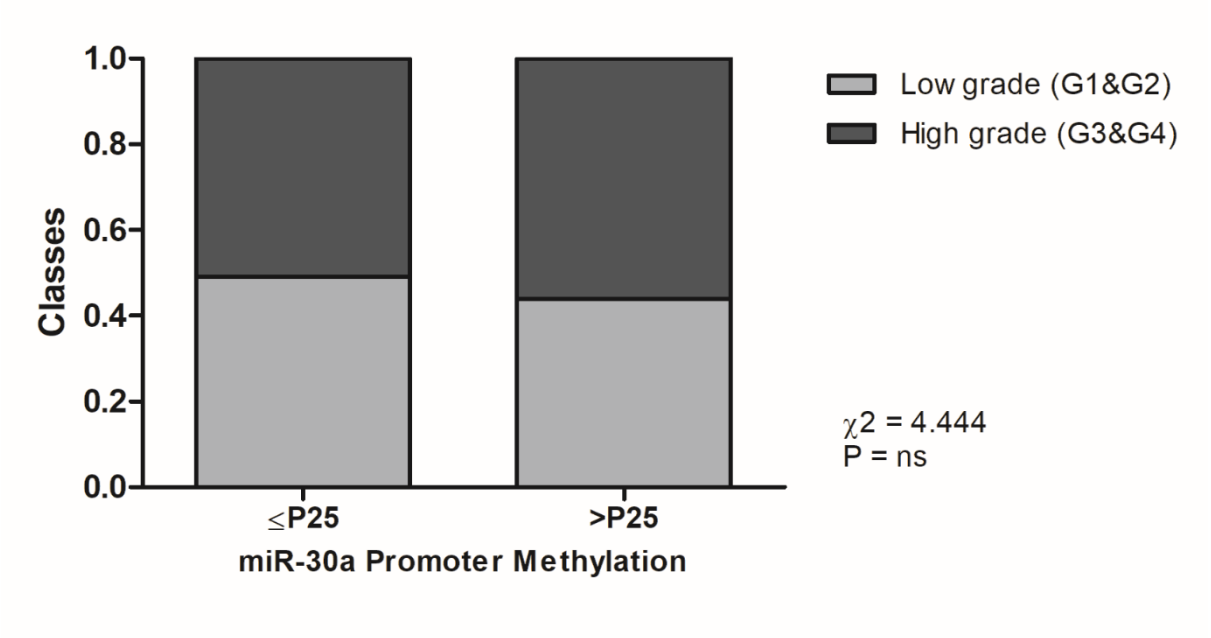

Figure 6 - Distribution of 25th percentile of miRNA-30a methylation levels according to low and high Fuhrman grade. The Fuhrman grade 1 and 2 were categorized as Low Grade, and Fuhrman grade 3 and 4 were categorised as High grade.

Supplementary J

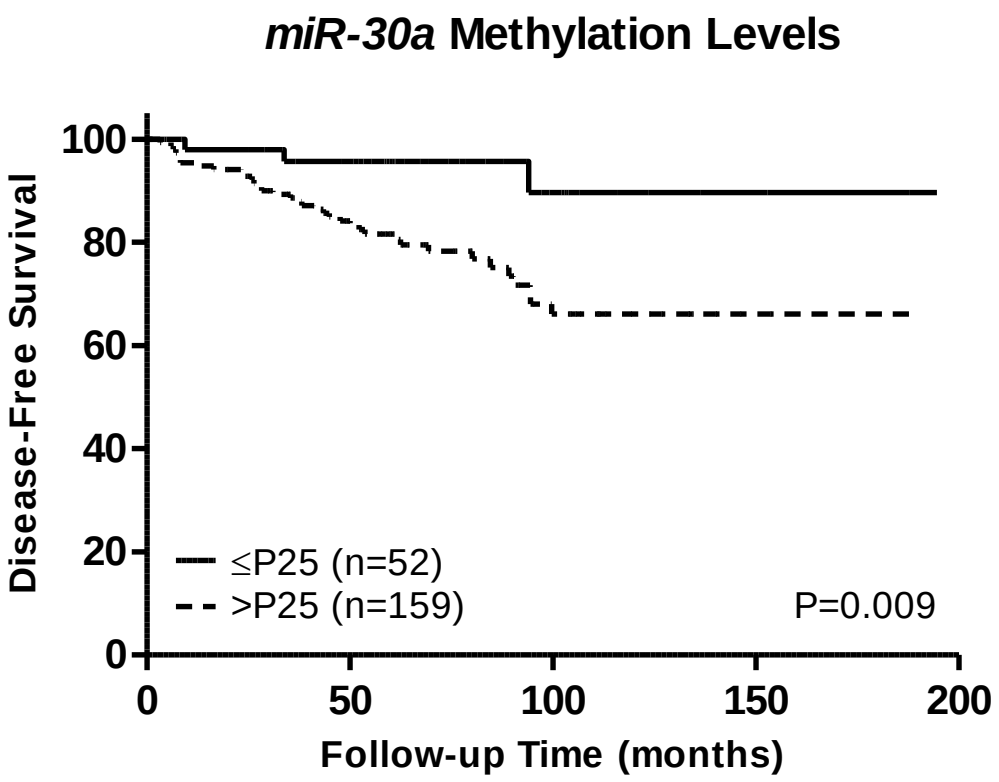

Figure 7 - Kaplain Meier estimated disease-free survival curve in ccRCCs for miRNA-30a methylation levels categorized using first quartil (25th percentile) value as cut-off.

Supplementary K

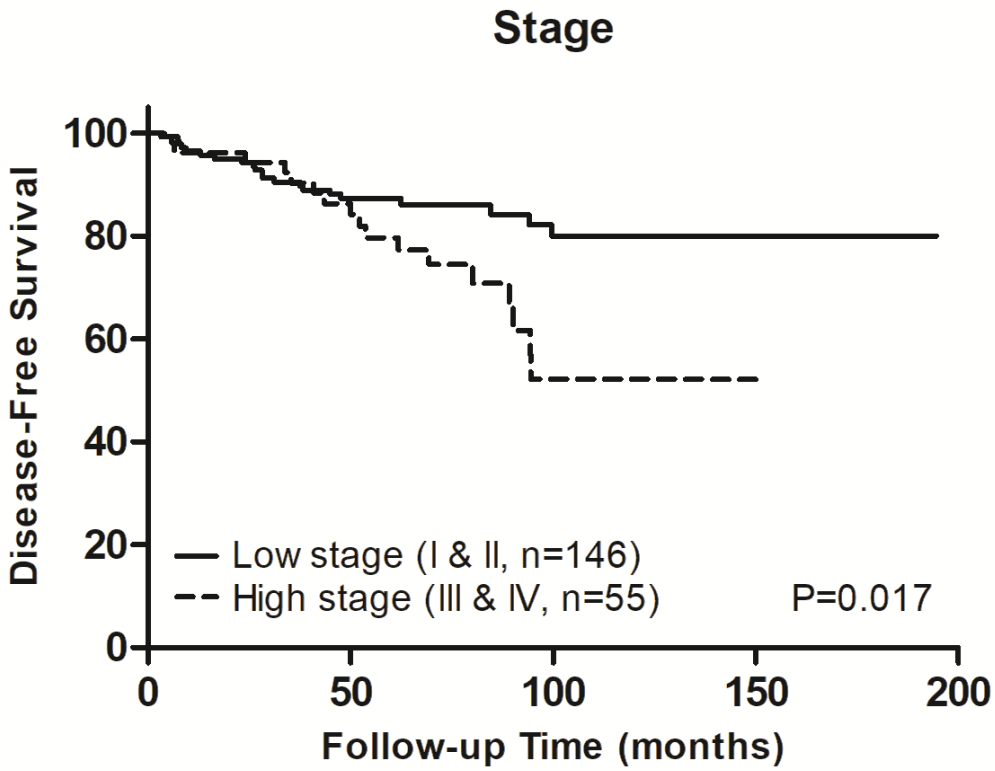

Figure 8 - Kaplain Meier estimated disease-free survival curve in ccRCCs for stage. The Stage I and II were categorized as Low Stage, and Stage III and IV were categorised as High stage.

Supplementary L

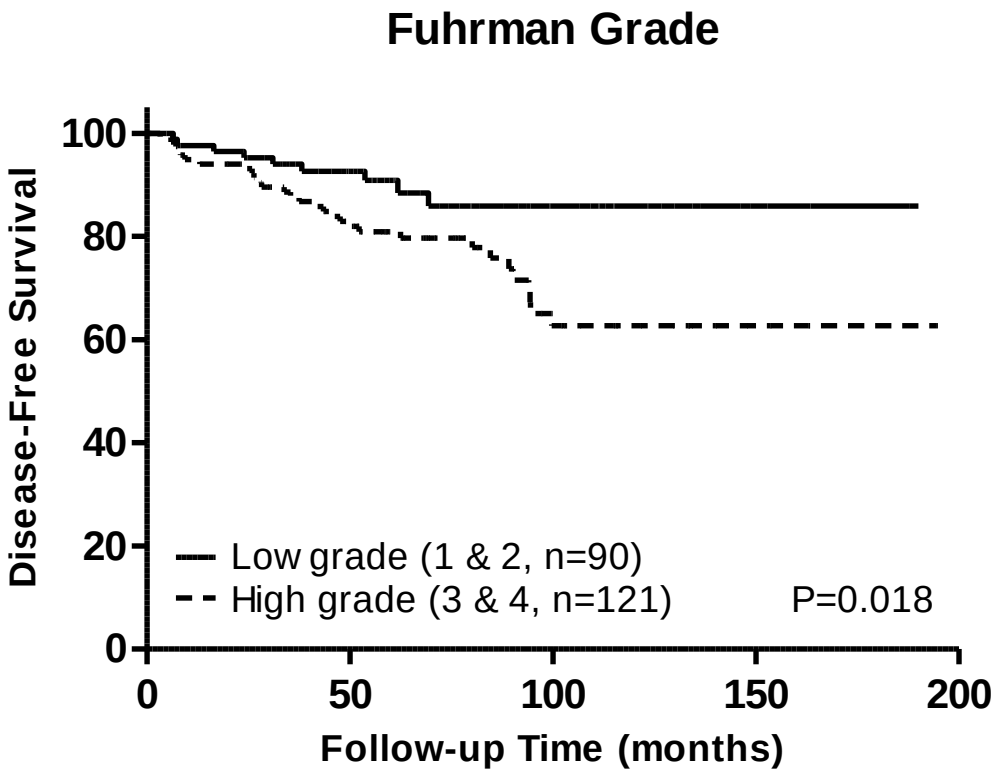

Figure 9 - Kaplain Meier estimated disease-free survival curve in ccRCCs for Fuhrman grade. The Fuhrman grade 1 and 2 were categorized as Low Grade, and Fuhrman grade 3 and 4 were categorised as High grade.