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The Role of HIF1-related Genes and Non-coding RNAs Expression in Clear Cell Renal Cell Carcinoma

STAMATIKI GRAMMATIKAKI[1], HECTOR KATIFELIS[1], KONSTANTINOS STRAVODIMOS[2], EMMANOUIL BAKOLAS[2], NIKOLAOS KAVANTZAS[3], DIMITRA GRIGORIADOU[3] and MARIA GAZOULI[1]

[1]*Laboratory of Biology, Medical School, National and Kapodistrian University of Athens, Athens, Greece;*
[2]*1st Department of Urology, National & Kapodistrian University of Athens, Laiko Hospital, Athens, Greece;*
[3]*2nd Department of Pathology, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece*

Abstract. *Background/Aim: Renal cell carcinoma is one of the three most common malignant urologic tumors, with clear cell renal cell carcinoma (ccRCC) representing its most common subtype. Although nephrectomy can radically cure the disease, a large percentage of patients is diagnosed when metastatic sites are present and thus alternative, pharmaceutical approaches need to be sought. Since HIF1 up-regulates the transcription of genes that range from metabolic enzymes to non-coding RNAs, and is a key molecule of ccRCC pathogenesis, this study aimed to investigate the expression ALDOA, SOX-6, and non-coding RNAs (mir-122, mir-1271, and MALAT-1) in samples from ccRCC patients. Patients and Methods: Tumor and adjacent normal tissue samples from 14 patients with ccRCC were harvested. Expression of ALDOA, mir-122, mir-1271, and MALAT-1 mRNA was estimated using real time PCR, whereas the expression of SOX-6 protein was investigated using immunohistochemistry. Results: Up-regulation of HIF1 was observed, accompanied with up-regulation of ALDOA, MALAT-1, and mir-122. On the contrary, the expression of mir-1271 was found to be reduced, a finding that can be attributed to a potential MALAT-1 sponge function. Furthermore, SOX-6 protein levels (a transcription factor with tumor suppressing properties) were also reduced. Conclusion: The observed dysregulated expression levels highlight the importance of ALDOA, MALAT-1, mir-122, mir-1271, and SOX-6, which remain less studied than the known and well-studied HIF1 pathways of VEGF, TGF-α, and EPO. Furthermore, inhibition of the up-regulated ALDOA, mir-122, and MALAT-1 could be of therapeutic interest for selected ccRCC patients.*

Correspondence to: Maria Gazouli, Ph.D., Professor, Laboratory of Biology, Medical School, National and Kapodistrian University of Athens, Michalakopoulou 176, Goudi, Athens, 11527, Greece. Tel/Fax: +30 2107462231, e-mail: mgazouli@med.uoa.gr

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Renal cell carcinoma is one of the three major urologic cancers with a steadily increasing incidence over the past decades, affecting more than 400,000 individuals annually at a global scale. Clear cell renal cell carcinoma (ccRCC) represents the most common subtype of renal malignancies and is characterized by a poor prognosis (1). Even though ccRCC can be treated surgically, most ccRCC cases are diagnosed when metastasis is already present with a median survival no greater than 13 months (2).

A key molecule in the development of ccRCC (3) and of several other malignancies is hypoxia inducible factor 1 (*HIF1*) whose expression is often dysregulated (4, 5). *HIF1* is involved in a plethora of cellular pathways and better understanding of these networks could be translated into inhibitors that would be of use as treatment for patients that either have metastases or that are not eligible for surgery.

One of *HIF1* targets, Aldolase A (fructose-bisphosphate aldolase a, *ALDOA*), is a ubiquitous glycolytic enzyme that drives the glycolytic metabolic pathway in mammalian cells (6) and is responsible for catalyzing the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (7). The hypoxic tumor environment boosts glycolytic enzymes *via HIF1* and increased expression of *ALDOA* is reported in malignancies, implying enhanced glycolysis in cancer cells, and thereby is considered to act as an oncogene (8, 9). Elevated levels of *ALDOA* expression predict poor survival in patients with ccRCC (10) and are related with metastasis and invasion of renal tumors (11).

A second, important target, is *MALAT-1* (also known as *NEAT1*) (12), a long noncoding RNA (lncRNA) that is well known for its role in malignancies. Although initially used

Table I. *Primer sequences*.

Gene	Forward primer	Reverse primer
ALDOA	5'ATGCCCTACCAATATCCAGC3'	5'GACAGCCCATCCAACCCT3'
HIF-1	5'CATAAAGTCTGCAACATGGAAGGT3'	5'ATTTGATGGGTGAGGAATGGGTT3'
MALAT-1	5'GAATTGCGTCATTTAAAGCCTAGTT3'	5'GTTTCATCCTACCACTCCCAATTAAT3'
GAPDH	5'CATCTCTGCCCCCTCTGCTG3'	5'GCCTGCTTCACCACCTTCTTG3'
Mir-122	5'ACACTCCAGCTGGGTGGAGTGTGACAA3'	5' TGGTGTCGTGGAGTCG 3'
Mir-1271	5' CTAGACGTCCAGATTGAATAGAC3'	5'GTCCGAGCTTGGTCAGAATG3'
snU6	5'ATTGCAACGATACAGAGAAGATT3'	5'GGAACGCTTCACGAATTTG 3'

as a prognostic biomarker for the prognosis and presence of metastatic sites in patients with non-small cell lung carcinoma, its role has well expanded beyond that. Although the exact mechanism of *MALAT-1* action remains unknown, it is widely accepted that increased levels of *MALAT-1* are linked to tumor cell proliferation and metastatic potential (13). Furthermore, it has been shown that MALAT-1 binds to *mir*-1271 and act as a molecular sponge [a term that refers to the binding of a lncRNA to a miRNA to inhibit its function (14). This is an important step during tumorigenesis since *mir-1271* inhibits epithelial to mesenchymal transition as well as tumor invasion (15).

Another significant target of *HIF1* is *mir-122*, which has been shown to play a role in promoting tumorigenesis. This can occur *via* several mechanisms including the PI3K/Akt pathway (16) and by targeting occludin (17).

Finally, *SOX-6* has been reported (18, 19) to be a gene target of both *mir-122* and *mir-1271*. Thus, this protein represents an indirect target of *HIF1* (*via mir-122* and *mir-1271*) with an important biological function due to its tumor suppressive properties. Indeed, research has shown that SOX-6, is essential for several developmental processes and is involved in the carcinogenesis of various malignancies. Current literature suggests its role in cellular differentiation, while reduced expression of *SOX-6* has been linked to a plethora of malignancies including liver and colorectal cancer (20).

Taken together, the aforementioned molecules (*ALDOA, mir-122, MALAT-1, mir-1271,* and *SOX-6*) could comprise a pathway that mediates crucial cellular events during oncogenesis. Thus, the aim of this study was to investigate the expression changes of the involved genes, which will contribute to the better understanding of the mechanisms through which *HIF1* plays a role in ccRCC. Furthermore, the knowledge of these axes could be translated into novel pharmaceutical agents for the effective treatment of ccRCC patients.

Patients and Methods

Patients and specimens. Renal cancer samples were collected from patients (n=14) who underwent radical or partial nephrectomy. The surgical operations were performed at the Laiko General Hospital of Athens over the past two years (2021-2022). The extracted kidney tissue specimens were collected and then transferred at the Laboratory of Biology (Medical School, National and Kapodistrian University of Athens) and stored at –80˚C. Normal adjacent tissue was also collected and used as control. The diagnosis was confirmed histologically. Fuhrman grading and TNM classification systems were used for histological classification (21). All subjects involved in this study gave their informed consent prior to participating and the present study was approved by the Ethics Committee of the Hospital.

Table II. *Patient demographics and TNM/Fuhrman grading*.

Variables			
Sex	Male	Female	
	6/14	8/14	
Age	64.5±10.95	62.2±9.657	
TNM staging	III	IV	
	9	5	
Fuhrman Grade	1	2	3
	3/14	4/14	7/14

RNA extraction and cDNA synthesis. Total RNA was extracted from cancer and adjacent normal tissues of the patients using NucleoZOL (Macherey-Nagel, Düren, Germany). The TAKARA kit (Takara Bio Europe SAS, Saint-Germain-en-Laye France) was used for cDNA synthesis from total RNA. All reactions were held on Thermal Cycler (Kyratec, SuperCycler, Queensland, Australia). The reaction conditions were as follows: 37˚C for 30 min and 85˚C for 5 min to deactivate reverse transcriptase.

Real-time PCR and gene expression analysis. To perform real time PCR, the KAPA SYBR FAST qPCR mix (KAPA BIOSYSTEMS, Cape Town, South Africa) was used. *GAPDH* and *U6* were used as genes of reference. The sequences of *HIF1, ALDOA, MALAT-1, GAPDH, miR-1271*, *mir-122,* and *U6sn* primers are illustrated in Table I. All reactions were held in duplicate to ensure reproducibility and gene expression was normalized to the expression of housekeeping genes. The reactions were held at SaCycler-96 (Sacace Biotechnologies, Como, Italy). *GAPDH* (*HIF1, ALDOA, MALAT-1*) and *U6sn* (*mir-122* and *mir-1271*) were used for within sample normalization. Fold change was calculated as $2^{-\Delta\Delta Ct}$ and is presented as fold regulation. Down-regulated genes

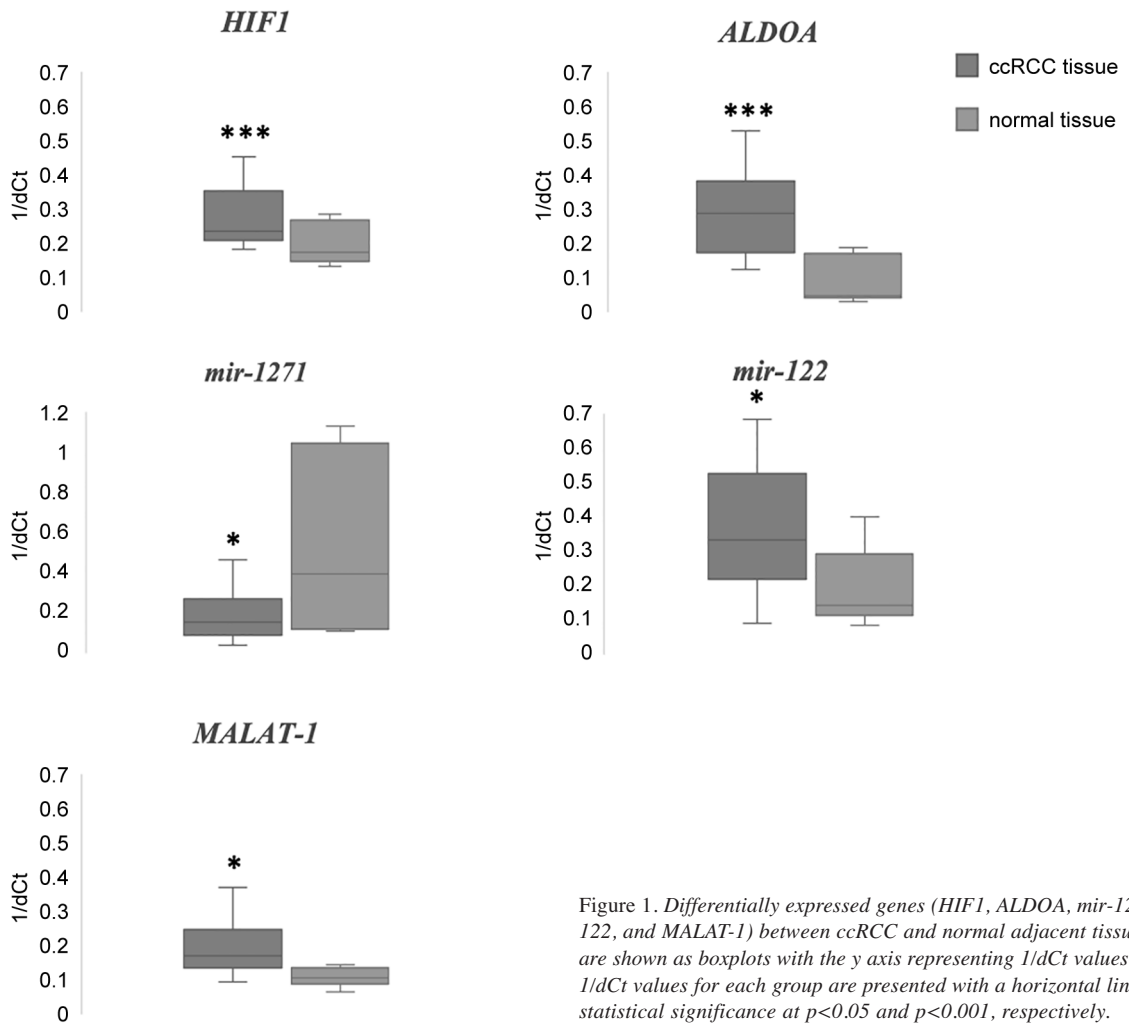

Figure 1. *Differentially expressed genes (HIF1, ALDOA, mir-1271, mir-122, and MALAT-1) between ccRCC and normal adjacent tissues. Data are shown as boxplots with the y axis representing 1/dCt values. Median 1/dCt values for each group are presented with a horizontal line. *, *** statistical significance at p<0.05 and p<0.001, respectively.*

are shown as the negative inverse of fold change and up-regulated as the fold change, as previously described (22).

Immunohistochemistry and investigation of SOX-6 expression. Briefly, sections from kidney samples (ccRCC and adjacent tissue) were fixed with 10% neutral buffered formalin solution for 24 h at room temperature and then dehydrated in a graduated ethanol series and embedded in paraffin. Then, sections were deparaffinized in xylol at 60˚C for 20 min and then hydrated in successive ethanol washes. Next, antigen retrieval was performed by incubating in citric acid for 15 min and then sections were washed 3 times with TBS. Kidney sections were incubated with 5% BSA-TBS for 1 h to reduce non-specific binding of the Fc part of primary/secondary antibodies. After being blocked, sections were incubated with the primary antibody anti-SOX-6 (ab30455, Abcam, Cambridge, UK) at a dilution 1:500 overnight at 4˚C. The following day, slides were washed in TBS and incubated with HRP-polymer (secondary antibody; DAKO, Glostrup, Denmark) for 15 min. Finally, and after TBS washes, 100 μl of DAB substrate was added for 1 min and then hematoxylin for 1 min.

Expression of SOX-6 protein was scored by two independent blinded observers using a classification system of three categories (23) that consists of 1+ (less than 10% positive cells), 2+ (positive cells between 10% and 50%) and 3+ (for more than 50% of cells positive).

Statistical analysis. All statistical analyses were performed using GraphPad version 3.00 (GraphPad Software, San Diego, Ca, USA). For comparison of gene expression between ccRCC and adjacent normal tissue *p*-values were calculated based on a Student *t*-test of the replicate $2^{-\Delta Ct}$ values for each gene between the two groups. $p<0.05$ was considered significant.

Results

The demographic and histological scoring are shown in Table II. The median age of patients was 64.5±10.95 years.

Altered gene expression is observed in ccRCC tissues. To test the hypothesis that *HIF1* expression can be related to

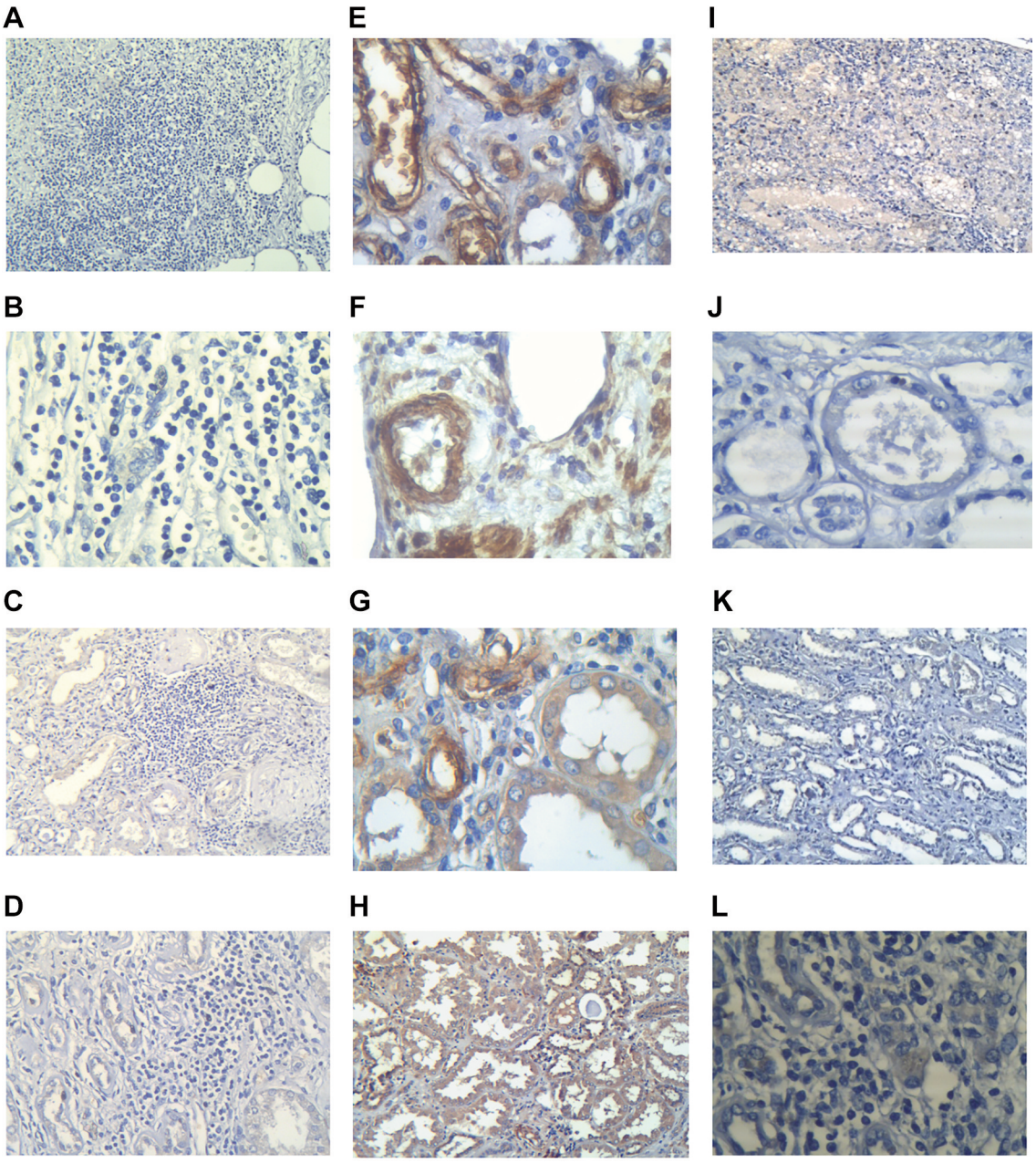

Figure 2. *SOX-6 expression was assayed using immunohistochemical staining in representative examples of ccRCC and normal tissue. Panels A-D refer to ccRCC tissue samples and panels E-H to normal tissue (with the primary antibody anti-SOX-6 (ab30455, Abcam). Panels I-L show the background noise (incubation only with HRP polymer) in ccRCC (I, J) and normal (K, L) tissue.*

the expression of *ALDOA*, *MALAT-1*, *mir-122,* and *mir-1271,* we investigated their expression in ccRCC samples and their adjacent tissues. The expression of *HIF1* was found to be up-regulated (1.4-fold change, $p<0.001$). Subsequently we investigated the fold change in the expression of *ALDOA*, *MALAT-1*, *mir-122,* and *mir-1271*. *ALDOA*, *mir-122,* and *MALAT-1* were found to be up-regulated. Compared to the adjacent tissues, their expression was up-regulated by 2.7-fold ($p<0.001$), 1.9-fold ($p<0.05$), and 1.7-fold ($p<0.05$), respectively.

On the contrary, the mRNA of *mir-1271* showed a decrease (-3.6-fold change, $p<0.05$), compared to adjacent tissue. The results are shown in Figure 1. A possible correlation between TNM stage or Fuhrman score and gene

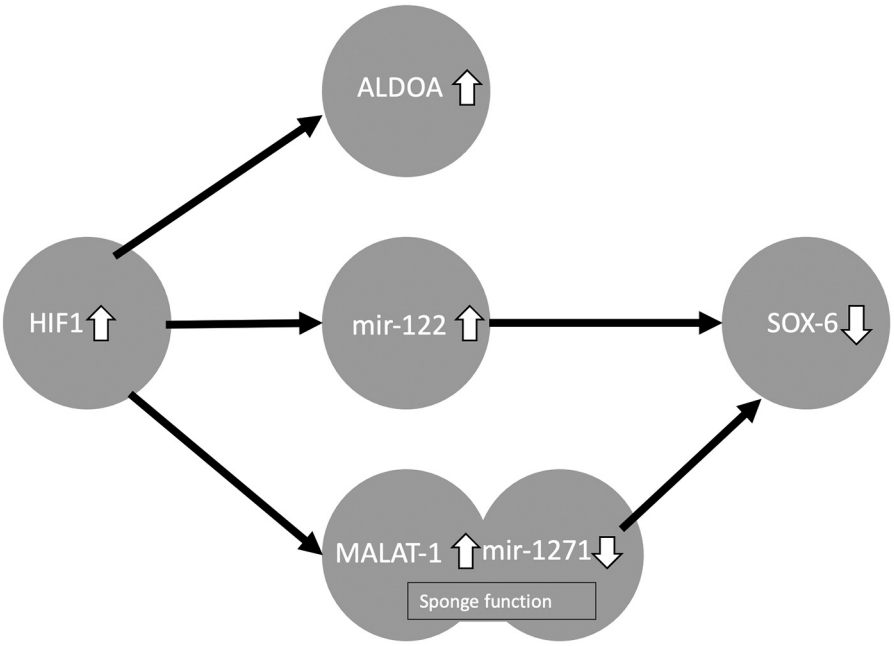

Figure 3. *The hypothesized HIF1 pathway based on our results. White arrows indicate the observed gene expression changes compared to adjacent tissue.*

expression changes was investigated. However, the number of samples per stage was not adequate to reach any statistically significant conclusions.

SOX-6 protein levels are reduced in ccRCC samples. Additionally, we studied the expression of SOX-6 protein. As illustrated in Figure 2, ccRCC tissue samples showed decreased expression (panels A-D) when compared to adjacent tissue (panels E-H). Of the 14 ccRCC tissue samples, approximately 90% were classified as 1+ and 10% as 2+. On the contrary, the adjacent tissues showed higher protein expression levels; approximately 60% were classified as 2+ and 40% as 3+. Regarding the site of SOX-6 expression, it was mainly observed in the cytoplasm and secondarily in the nuclei of the cells.

Discussion

It is well known that *HIF1* is a key molecule in glycolysis and cancer, allowing malignant cells to boost anaerobic metabolism when the available oxygen is limited (24). Based on our results, *HIF1* was found to be up-regulated. This finding agrees with current literature; not only *HIF1* has been repeatedly found to be up-regulated in malignancies but it also indicates a poor prognosis (25). Its cancer-promoting actions affect several targets including glycolytic enzymes (*ALDOA*) and non-coding RNAs (including *mir-122* and *MALAT-1*) (26-28). However, unlike the well characterized *VEGF, TGF-α,* and *EPO* pathways (29), the connection between HIF1 expression and *mir-122, MALAT-1,* and *ALDOA* remains poorly studied.

ALDOA, a glycolytic enzyme targeted by *HIF1* has been also reported to play a significant role in malignancies (30). Based on current literature, high levels of *ALDOA* promote tumor growth and metastatic potential (31). Similarly to *HIF1*, we found *ALDOA* to be up-regulated, a finding that is in accordance with two studies (10, 11) that were performed in Asian populations concerning ccRCC. Despite the lack of additional studies in ccRCC, literature shows that increased expression of ALDOA is linked with reduced survival in several malignancies (31, 32).

Regarding the role of non-coding RNAs, *mir-122*, targets *HIF1* (33) and has been studied in a plethora of cancers. Whether its up-regulation boosts or inhibits tumorigenesis/invasion is a matter of dispute (34, 35). In our study, the expression of *mir*-122 was found to be increased in ccRCC samples. *Mir-122* up-regulation in RCC was also observed in two other studies, which showed that increased tissue levels of *mir*-122 promote tumorigenesis by targeting the *SRY2* and *PI3K/Akt* pathway (16, 33). However, a third study suggested that increased serum levels of *mir-122* serve as a prognostic marker of RCC (36). Interestingly, Grimm *et al*. (37), reported that *mir-122* high expression leads to reduced levels of SOX-6, a transcription factor with tumor suppressive function. Thus, our results support the hypothesis that *mir-122* promotes RCC not only through the SRY and

PI3K/Akt pathways but also *via* SOX-6, highlighting a potential therapeutic role for *mir*-122 inhibitors.

Additionally, *MALAT-1* is also involved in the *HIF1* pathway (38). Increased expression of *MALAT-1* is a common finding in several malignancies and has been connected to reduced patient survivorship. Recently, Liu *et al*. (14), showed that one of the mechanisms of action of *MALAT-1* is the decrease of *mir-1271* levels in multiple myeloma *via* a sponge function. This mechanism could also occur in ccRCC, since in accordance with a previous study (14) in our results, the up-regulation of *MALAT-1* is also followed by the down-regulation of *mir-1271*. Thus, our results suggest, that at least one of the roles of *MALAT-1* in ccRCC lies on the reduction of *mir-1271*, which canonically acts as tumor suppressor miRNA (39). Only one other study has investigated the potential *of mir-1271* as a urinary biomarker but did not find any statistically significant changes in its urine levels (40). To the best of our knowledge, this is the first study to show the reduction of *mir-1271* in ccRCC patient samples.

Finally, we evaluated the SOX-6 protein levels and our results showed decreased levels of SOX-6 protein in cancer tissue compared to the control samples. Only 3 other studies (41-43) have evaluated the role of SOX-6 in ccRCC, yet none of them was performed on Caucasian populations. These studies also showed SOX-6 down-regulation and that its effects are mediated *via* the regulation of the Wnt/β-catenin pathway. Moreover, decreased levels of SOX-6 have been reported in cervical, prostate, pancreatic and breast cancer (44-47), findings supporting the universal role of SOX-6 in carcinogenesis.

It should be noted that our study is not exhaustive regarding the study of all miRNAs that target SOX-6. Apart from *mir-122* and *mir-1271*, several other miRNAs target SOX-6 (such as *miR-208b* and *miR-499*) (47). The focus of this study was the HIF pathway and thus we selected to study *mir-122* and *mir-1271* because they participate in this pathway (18, 19).

Collectively, our results support the hypothesis that there is a connection between HIF1 and ALDOA, *mir*-122, *mir*-1271, MALAT-1, and SOX-6 as shown in Figure 3. It should be noted that there are two limitations to the present study: the small number of samples and the fact that the samples were obtained at a single hospital. However, our results showed homogenous clustering with limited intra-group (ccRCC and adjacent tissue) variation, a fact that minimizes the potential impact of the sample size.

To conclude, *HIF1* seems to be involved in the up-regulation of *ALDOA*, *mir-122,* and *MALAT-1*, all of which have been found to promote cancer-related properties. On the contrary, *mir*-1271, which has tumor suppressive properties, was reduced possibly *via* the sponge action of *MALAT-1*. Finally, *HIF1* up-regulation could indirectly (*via mir*-122) down-regulate the tumor suppressive SOX-6 protein. These findings not only indicate a potential pathway, but also could propose the inhibition of these molecules for patients that are not eligible for nephrectomy (including patients with metastatic disease) in the treatment of ccRCC.

Conflicts of Interest

The Authors have no conflicts of interest to disclose in relation to this study.

Authors' Contributions

Conceptualization: S.G., H.K. and M.G.; data curation: E.B. and MG; methodology: S.G. and H.K.; supervision: K.S., N.K. and M.G.; original draft: S.G., H.K. and D.G.; writing, review and editing: K.S. and M.G. All Authors have read and agreed to the published version of the manuscript.

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