

Autophagic Gene Polymorphisms in Liquid Biopsies and Outcome of Patients with Metastatic Clear Cell Renal Cell Carcinoma

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Abstract. Background/Aim: Autophagy has been shown to be involved in cancer development and response to cancer therapy. In this study, genotypes of autophagic genes were analyzed to assess their correlation with the risk of clear cell renal cell carcinoma (ccRCC) and the outcome of patients treated with pazopanib for metastatic ccRCC. Materials and Methods: Single nucleotide polymorphisms (SNPs) were selected in the following genes: ATG4A (rs7880351), ATG4B (rs6709768), ATG4C (rs2886770, rs6670694, rs6683832), ATG5 (rs9373839, rs3804333, rs490010), ATG16L1 (rs6752107), ATG16L2 (rs10751215) and IRGM (rs10059011). The Kaplan–Meier method and log-rank test were used to evaluate differences between groups. Results:

Forty patients with metastatic ccRCC treated with pazopanib were included in the analysis. ATG16L2 rs10751215 was significantly less frequent in patients with ccRCC compared to the general population, suggesting its potential protective role, while ATG4A rs7880351, ATG4C rs6670694 and rs6683832 and ATG5 rs490010 were correlated with the progression-free survival (PFS) of patients treated with pazopanib. Conclusion: Our results suggest, for the first time, that autophagic gene SNPs are associated with ccRCC risk and patient outcome.

Renal cell carcinoma (RCC) is a malignancy arising from kidney parenchymal cells, with an incidence of approximately 3.7% and about 63,000 new cases expected in the United States in 2016 (1). Curative surgery, whenever possible, is the mainstay for localized or locally advanced forms. Nearly 20-25% of patients (depending on stage at diagnosis) will develop recurrent or metastatic disease after radical surgery, whilst 25-30% of patients present with metastatic disease (2, 3).

In the past years, we have witnessed a dramatic revolution in the therapeutic armamentarium of RCC, due to the

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introduction of novel target agents such as tyrosine kinase inhibitors (TKIs) sorafenib (4), sunitinib (5), pazopanib (6), axitinib (7) and cabozantinib (8) and mammalian target of rapamycin (mTOR) inhibitors temsirolimus (9) and everolimus (10). These drugs present considerable variations among their mechanisms of actions (11-16), that can explain the differences observed in terms of objective response rate (ORR), progression-free survival (PFS) and tolerability (4-10).

The autophagy consists in the degradation or the recycle of cytosolic components harmful to the cell or no more needed through the formation of a double membrane-layered autophagosome enwrapping cell constituents. When autophagosomes fuse with lysosomes (forming autophagolysosomes), the enzymatic activity leads to catabolism of sequestered components (17). In healthy cells, autophagy plays a homeostatic role for cell survival in stress conditions, nevertheless alterations in this process may cause cell death for “incomplete autophagy” or due to extreme vesicle storage (18). Autophagy related gene 4 (*ATG4A*), *ATG4B* and *ATG4C* are 3 of the 4 *ATG4* homologs (*ATG4A-D*) involved in autophagosome elongation (19). *ATG5* is activated by *ATG7* and forms complexes with *ATG12-ATG16L1* and *ATG12-ATG16L2* that participates in autophagosome elongation through the formation of autophagy-related light chain 3 (LC3)-II protein (19). As for IRGM, it regulates the formation of autophagy initiation complexes (19). It has been reported that this process is involved in modulating the activity of TKIs and mTOR inhibitors in RCC (13, 19, 20).

It is interesting to note that sunitinib is a lysosomotropic agent; therefore, it is accumulated inside lysosomes inducing structural and functional alterations (13). In this manner, sunitinib is able to compromise autophagolysosomes generation and lysosomal enzymatic activity leading to “incomplete autophagy” in RCC cells (13). On the other hand, pazopanib can interfere with membrane trafficking (autophagosome formation and fusion with lysosomes) leading to accumulation of vesicles in the cell cytoplasm (13). As for everolimus, autophagy has been shown to be involved in the mechanisms of primary and acquired resistance to this drug in RCC models (13, 16-19).

In this study, we analyzed genotypes of some genes implicated in the autophagic process, aiming to verify whether they correlated with increased tumor risk and/or with the outcome of patients treated with first-line pazopanib for metastatic RCC.

Materials and Methods

Study population. The study population consisted of all consecutive patients aged 18 years or older treated with pazopanib for metastatic RCC in 6 Italian Institutions between 2011 and 2016. Other inclusion criteria included Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2 ; adequate organ function; no serious concomitant diseases. Patients were included consecutively to avoid

Table I. *Patients' characteristics.*

Chaeacteristics	Patients, N (%) (n=40)
Median age, y (range)	65 (43-81)
Gender	
Male	23 (58)
Female	17 (42)
Tumor histology	
Clear cell	40 (100)
ISUP Grading	
1	3 (7)
2	12 (30)
3	13 (32)
4	5 (13)
Unknown	7 (18)
Vascular infiltration	
Yes	11 (28)
No	25 (62)
Unknown	4 (10)
Disease status at RCC diagnosis	
Metastatic	10 (25)
Non-metastatic	30 (75)
Radical nephrectomy	
Yes	36 (90)
No	4 (10)
ECOG-PS ≥ 2	4 (10)
MSKCC risk group	
Good	15 (38)
Intermediate	22 (56)
Poor	3 (6)
Sites of metastases	
Lung	26 (65)
Lymph node	18 (45)
Liver	4 (10)
Bone	7 (18)
Brain	1 (3)

selection bias and were treated with pazopanib as first-line therapy according to the local guidelines of each participating Center. Pazopanib was continued until clinical and/or radiological progression or unacceptable adverse events or death. Follow-up was performed by physical examination, periodical laboratory analyses and computed tomography (CT) or magnetic resonance imaging (MRI) scans every 8-12 weeks.

Written informed consent was obtained from each subject and another consent for the biological procedures was obtained by alive patients. This study was carried out in accordance with the approval by the Ethical Committee of the institutions included in this study.

Single nucleotide polymorphism (SNP) selection, DNA extraction, genotyping and predictions. SNPs in the above-mentioned genes were selected using National Center for Biotechnology Information (NCBI) data and reviewing medical literature, according to the following criteria: 1) polymorphisms located in biologically relevant areas of the gene (*i.e.* intron, 5' UTR and 3' UTR or promoter region); 2) minor allele frequency (MAF) $\geq 10\%$; 3) the genetic polymorphism was established and well documented.

Table II. Position in the gene and base exchange of polymorphism studied group.

Sample ID	NCBI SNP reference	Assay ID	Reporter 1 Dye	Reporter 1 Sequence	Reporter 1 Frequency	Reporter 2 Dye	Reporter 2 Sequence	Reporter 2 Frequency	Context sequence
ATG4A	rs7880351	C_266607_10	VIC	G	0.63	FAM	C	0.37	GCATAAGGGTTGCA ATCAAGAAAGG(G/C) AGCACCAGGCTTG GATGAAGAAAC
ATG4B	rs6709768	C_313966_10	VIC	C	0.44	FAM	G	0.56	ACTTTTGCACAGGTC TGCTTGGTTA(C/G) TGAAAGCATGTCTC CCTTTCTAGGA
ATG4C	rs2886770	C_15840433_10	VIC	A	0.38	FAM	G	0.62	CTGTTGAACCTCTTTT CTTCTCTGTG(A/G) TTCCAAGTGTCTT GATTATATACAT
	rs6670694	C_1767372_10	VIC	A	0.45	FAM	G	0.55	ATTGTGCATAAGGA GACCTTATACT(A/G) CAAAAAGATTTGC AAAAAGTAGATG
	rs6683832	C_30152455_20	VIC	A	0.62	FAM	G	0.38	ATTTAATACAAGATT CTTAAACTTG(A/G) TTCTGTCTCTAT TATTTAATTCTA
ATG5	rs9373839	C_3001898_10	VIC	C	0.21	FAM	T	0.79	ATCACAACTTAAA TCAGGTTTGAG(C/T) CCTATGAGTTATA TACAGTTACATG
	rs3804333	C_1328945_10	VIC	C	0.79	FAM	T	0.21	TACCCAGAATTTTCC ACTGTGACCA(C/T) AGAAGGGTCATGA CTAAGGAATAAA
	rs490010	C_1328956_10	VIC	A	0.47	FAM	G	0.53	GTCCCCTCTGTACT TCCATATCCAC(A/G) TCTACATGCAAG ATTTTGTTTTTT
ATG16L1	rs6752107	C_9095450_10	VIC	A	0.58	FAM	G	0.42	TTCCCCTTACTGCTT GCAGCTTAAG(A/G) GTACAAAAATGTT TGACCTTTACC
ATG16L2	rs10751215	C_2064588_10	VIC	C	0.64	FAM	T	0.36	GGCTGTGCATCTCAG GCCTTGCCGC(C/T) GACAGGCGACGCT GGCACAGGCTTA
IRGM	rs10059011	C_30052989_10	VIC	A	0.49	FAM	C	0.51	CTCGGGGGTCAAAGG CTGGTGGCTT(A/C) CTTCACGTATATTG CAGCATTTTCAG

The genomic DNA of patients was extracted from blood, using the DNA Blood Mini kit according to the manufacturer's instructions. Genotyping of polymorphisms was performed using pre-designed TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Amplifications and analysis were carried out on the 7300 Real-Time PCR System (Applied Biosystems), using the SDS software v1.4.0 for allelic discrimination (Applied Biosystems). About 10% of samples were randomly remade for genotype confirmation and the results were 100% concordant. Data from general CEU population were provided by the HapMap project (www.HapMap.org). When these data were not available we considered the frequencies reported in the 1000 genome project (www.1000genomes.org).

The genotype frequencies of ATG4A, ATG4B, ATG4C, ATG5, ATG16L1, ATG16L2 and IRGM were checked for the Hardy–

Weinberg equilibrium (HWE) and linkage disequilibrium (LD), calculated using the Haploview 4.2 (Broad Institute, Cambridge, MA, USA). The most common genotypes in control subjects were considered as references.

To assess if a polymorphism can alter 5' and 3' splice sites, both genuine and cryptic, the NNSPLICE tool of the BDGP consortium was used (21). This tool uses an artificial neural network (ANN) algorithm trained with experimentally assessed data to estimate the splicing sites strength. Indeed, the higher the strength is, the higher the probability that the sequence is a splice site. To detect splice sites with higher sensitivity, Human Splicing Finder 3.0 was also used (22). Detailed indications for the interpretation of the consequences of the splicing site alteration events were previously reported (23).

SpliceAid2 tool has been used in order to predict if polymorphisms can alter binding sites for splicing regulatory factors

(<http://www.introni.it/spliceaid.html>) (24). This resource detects only experimentally assessed target RNA sequences in order to reduce the false positive results. To interpret the predicted gain or loss of splicing factor, it should be taken into account that i) the gain of exonic silencers within an exon is usually a severe event that can lead to a partial or a total skipping of the concerned exon, ii) the loss of exonic silencers within an exon could alter the inclusion rate of an alternative exon, iii) the loss or gain of exonic enhancers is usually tolerated because these motifs are abundant in exons. The list of the splicing enhancer and silencer regulatory factors was previously published (25).

Statistical analysis. Data were collected from electronic and paper charts by the local physicians and checked at the central data management. Overall survival (OS) was calculated as the time from the date of first diagnosis to death or last follow-up visit. First-line OS was calculated from the start of treatment with pazopanib for metastatic disease to death or last follow-up. Patients who died without progression were censored at the time of death, whilst patients lost to follow-up were censored at the time of last contact.

PFS was defined as the interval from the start of first-line therapy to disease progression or last follow-up or death. Disease-free survival (DFS) was defined as the time from surgery to disease recurrence. OS, PFS and DFS were evaluated with the Kaplan–Meier method and log-rank test were used to test differences between groups. A Cox-regression model was applied to the data with a univariate approach and used to assess the role of polymorphisms as prognostic factors. The association between categorical variables was assessed using the Fisher’s exact test. All significance levels were set at a 0.05 value. Statistical analysis was performed with the MedCalc statistical software package version 11.4.4.0 (MedCalc Software, Broekstraat 52, 9030 Mariakerke, Belgium).

Results

Overall study population. Clinical data of 40 patients with metastatic RCC treated in 6 Italian centers were retrospectively collected. Twenty-three of them were males (58%). Median age was 65 years (range=43-81 years). Tumor histology was clear cell in all patients. Thirty patients (75%) had localized RCC at the time of first diagnosis and were treated with pazopanib at recurrence, while ten patients (25%) were metastatic at presentation. The majority of included patients (90%) were treated with radical nephrectomy at first diagnosis of RCC. A diagnosis of metastatic ccRCC, prognostic categories using Memorial Sloan Kettering Cancer Center (MSKCC) criteria were good in 15 patients (38%), intermediate in 22 patients (56%) and poor in 3 patients (6%). The complete list of patient characteristics is shown in Table I.

Description of polymorphisms, Hardy–Weinberg equilibrium and linkage disequilibrium. Three SNPs were identified in *ATG4C* (rs2886770, rs6670694, rs6683832) and *ATG5* (rs9373839, rs3804333, rs490010). A single SNP was identified in *ATG4A* (rs7880351), *ATG4B* (rs6709768), *ATG16L1* (rs6752107), *ATG16L2* (rs10751215) and *IRGM* (rs10059011). Position in the gene and base exchange are

Table III. Hardy–Weinberg equilibrium of selected SNPs. HWpval of a polymorphism is defined as the probability that its deviation from the H-W equilibrium could be explained by chance.

Gene	Id SNP	Position	ObsHET	PredHET	HWpval
<i>ATG4C</i>	rs6683832	Chr 1: 62811233	0.5	0.483	1.0
	rs2886770	Chr 1: 62837115	0.475	0.485	1.0
	rs6670694	Chr 1: 62862187	0.41	0.492	0.426
<i>ATG5</i>	rs9373839	Chr 6: 106207742	0.35	0.349	1.0
	rs3804333	Chr 6: 106279340	0.325	0.335	1.0
	rs490010	Chr 6: 106292583	0.395	0.5	0.2896
<i>ATG4B</i>	rs6709768	Chr2: 241666621	0.55	0.5	0.8125
<i>ATG16L1</i>	rs6752107	Chr2: 233252802	0.4	0.495	0.3268
<i>IRGM</i>	rs10059011	Chr 5: 150847160	0.526	0.494	1.0
<i>ATG16L2</i>	rs10751215	Chr 11: 72815042	0.575	0.497	0.5524
<i>ATG4A</i>	rs7880351	Chr X: 108111552	0.529	0.5	1.0

shown in Table II. Based on values reported in Table III, no SNP was rejected. Figure 1 shows the pairwise linkage disequilibrium between any two polymorphisms of *ATG4C* and *ATG5* genes.

Genotyping and prognostic analyses. This study analyzed the SNP frequency of genes involved in autophagy in RCC compared with the general population. All frequencies and genotype distributions are shown in Table IV. The frequency of *ATG16L2* polymorphism rs10751215 was significantly lower in RCC than for general population (46% vs. 64%, $p=0.024$, Table IV). In addition, the frequency of *ATG16L1* polymorphism rs6752107 was lower in RCC than for general population (58% vs. 45%), although the difference was only slightly significant ($p=0.09$). As for the other autophagic genes, we did not observe significant differences (Table IV).

No significant correlation was found between selected SNPs and gender, age, Fuhrman grade, tumor stage or sites of metastases. Median OS from diagnosis was 265.3 months (95%CI=19.1-398.6). No significant differences were found based on gender or age (≥ 65 y vs. < 65 y). As for the prognostic role of SNPs on OS, patients with the *ATG4* rs7880351 genotype had a significantly shorter OS (although not reached in both groups) compared to C and GC genotypes ($p=0.03$, Figure 2).

Median OS from the start of pazopanib was 48.0 months (95%CI=9.1-53.4). The OS was longer in females, although the difference was not significant (not reached vs. 48.0 months, $p=0.078$). No significant differences were found based on age or MSKCC groups or on selected SNPs.

Nineteen patients (47.5%) experienced partial responses (PR), 1 (2.5%) complete response (CR), 14 (35.0%) had stable diseases (SD) and 6 (15%) progressive diseases (PD). Median PFS was 13.1 months (95%CI=3.9-37.6). No significant differences were found based on MSKCC group, gender or age

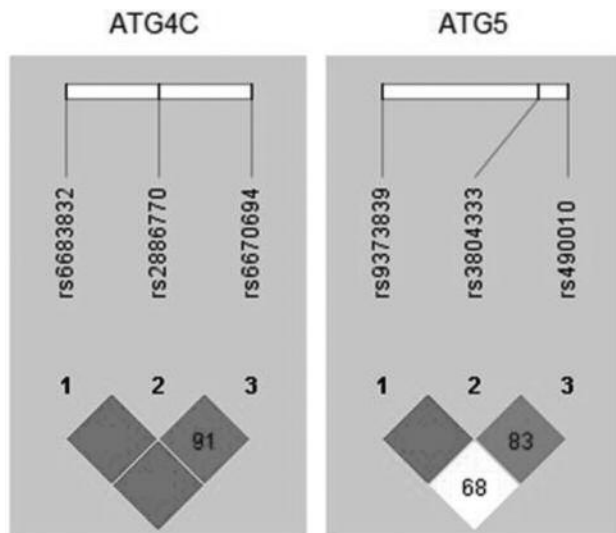


Figure 1. Linkage disequilibrium (D') among genetic polymorphisms of *ATG4C* and *ATG5* genes. *ATG4C* and *ATG5* SNPs are in a 51 Kb and 85 Kb region respectively. Number inside the squares represents the D' value expressed as a percent. Squares without numbers represent D' values of 1.0, that is complete linkage disequilibrium. Darker shades represent stronger linkage disequilibrium.

(≥ 65 y vs. < 65 y). Patients with *ATG4* *Ars7880351* G genotype (G for males, GG for females due to its location on X chromosome) had a significantly shorter PFS compared to females with CC and GC genotypes (16.7 vs. 23.9 months, $p=0.043$, Figure 3A). As for *ATG4C* rs6670694, patients with AA and GA genotypes had longer PFS compared to GG (23.9 vs. 13.1 months, $p=0.067$, Figure 3B), while patients with *ATG4C* rs6683832 AA had longer PFS than AG and GG (22.3 vs. 14.7 months, $p=0.110$, Figure 3C). In the studied population these two polymorphisms are in complete LD so they group into a haplotype block. It means also that, in our sample, we cannot establish if both or only one of them is causal (26).

Finally, patients with *ATG5* rs490010 GG genotype had shorter PFS compared to AA and AG (11.5 vs. not reached, $p=0.039$, Figure 3D).

No correlation was found between SNPs and the development of all-grade and high-grade adverse events. Of the 22 patients who progressed during first-line, 16 (73%) received a second line therapy, with a median PFS of 5.5 months (95%CI=2.9-10.2). Of them 14 (88%) were treated with everolimus and 2 (12%) with sorafenib.

Results from computational analysis. Here we report the results of the predicted molecular effects due to the polymorphism based on the predictions of SpliceAid2 tool. When not specified, we assumed that the nucleotide variation

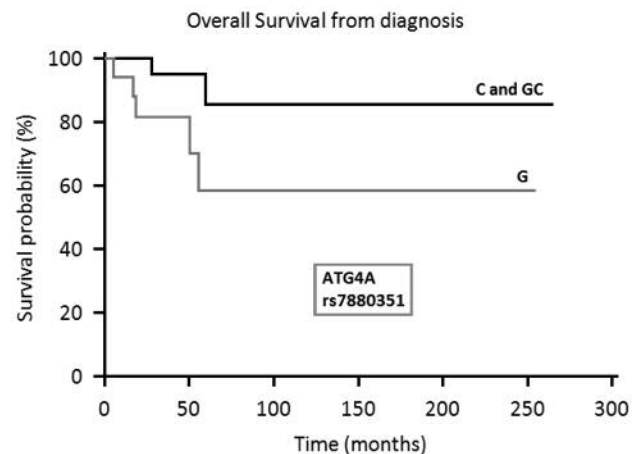


Figure 2. Overall survival according to *ATG4A* polymorphisms in patients treated with pazopanib for metastatic ccRCC.

lies within an intron, moreover only the predicted differences between alleles are reported in Figures 4 and 5.

rs7880351 polymorphism of the *ATG4A* autophagy related 4A, cysteine peptidase gene could create a binding site for SRp30c and SRp40 splicing proteins according to SpliceAid. HSF predicts that the C allele of rs7880351 causes the strengthening (score from 0.60 to 0.70) of a cryptic 3' splice site (caagaaaggcagCACCAGGCT). However, the score seems yet insufficient to activate the 3' splice site and provoke the inclusion of an ectopic exon during pre-mRNA splicing (Figure 4A).

rs6709768 variation of the *ATG4B* autophagy related 4B, cysteine peptidase gene lies within an intron, at 23bp upstream the 3' splicing site (ss). It reinforces four cryptic splice sites: a 5'ss (CTGCTTGgtagtga with score 0.98) according to NNSPLICE, two 5'ss (CTTGGTTAgtagaaagc with score 0.67 and TCTGCTTGgtagtga with score 0.88) according to HSF, a 3'ss (gcttggttagTGAAAGC with score 0.73) according to HSF. Moreover, a binding site for the hnRNP A1 exonic silencer is created. Altogether, since the scores of two 5' splicing sites is higher than 0.80 it is possible that during splicing one of these 5'ss is used and splicing alterations occur (Figure 4B).

rs2886770 of the *ATG4C* autophagy related 4C, cysteine peptidase gene only weakly reinforces a cryptic 5'ss (TCTGTGgttccaa), according to HSF, so no effect is expected (Figure 4C).

rs6670694 of the *ATG4C* autophagy related 4C, cysteine peptidase gene could destroy the binding sites for hnRNP DL and SRp40 and create the binding site for SRp20. Since the loss of the SRp40 exonic enhancer is compensated by SRp20 and many other exonic silencers, apart from hnRNP DL, bind the intron, we deduce that this variation has not molecular effects (Figure 4D).

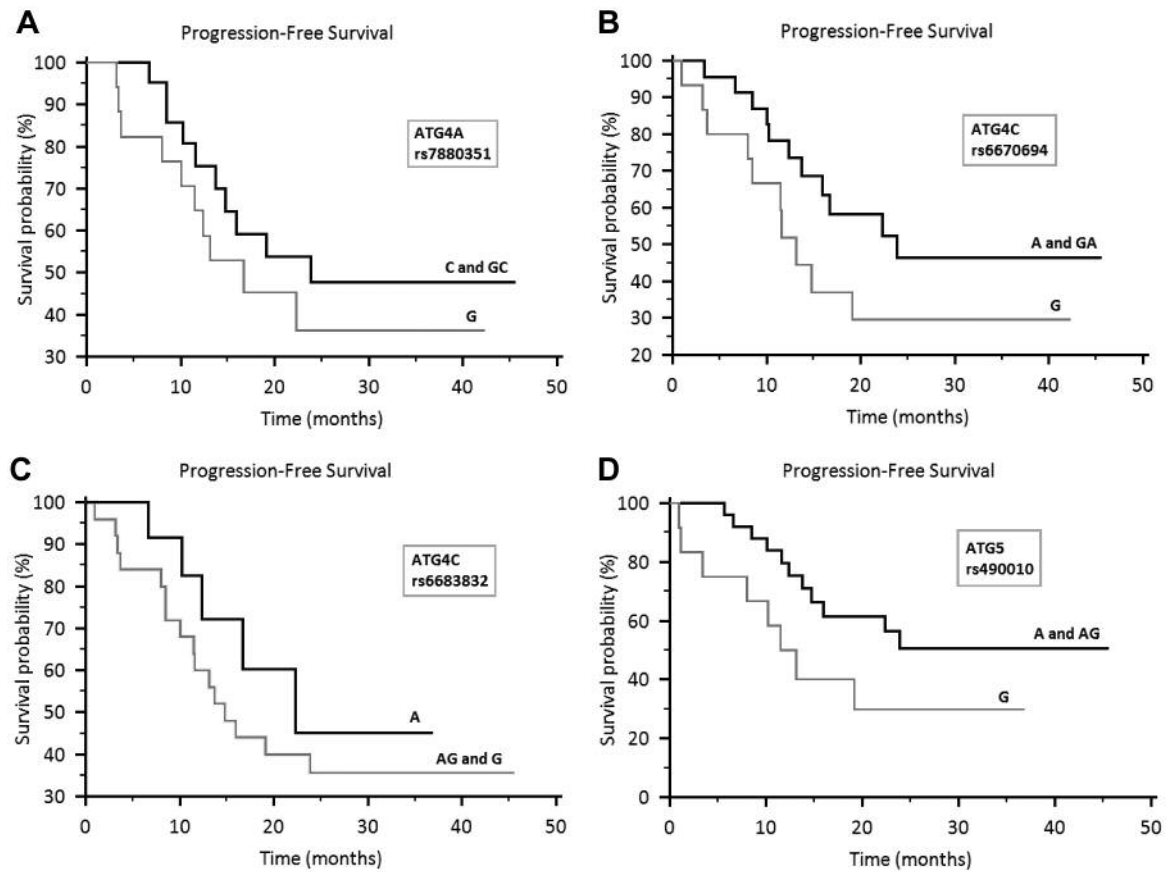


Figure 3. Progression-free survival according to ATG4A, ATG4C and ATG5 polymorphisms in patients treated with pazopanib for metastatic ccRCC.

Table IV. Genotype and allele frequencies of evaluated genes polymorphisms.

Gene	SNPs	Allele	Frequencies general population	Frequencies study cohort	n. sample	p-Value	Yates	Odds Ratio
ATG4A	rs7880351	G	63%	54%	40/40	0.2353	0.3047	1.47
		C	37%	46%				
ATG4B	rs6709768	G	56%	50%	40/40	0.4471	0.5472	1.27
		C	44%	50%				
ATG4C	rs2886770	G	62%	59%	40/40	0.6743	0.7959	1.15
		A	38%	41%				
ATG4C	rs6670694	G	55%	56%	39/39	0.8593	0.9871	0.94
		A	45%	44%				
ATG4C	rs6683832	A	62%	59%	38/38	0.7249	0.8525	1.12
		G	38%	41%				
ATG5	rs9373839	T	79%	78%	40/40	0.8181	0.9694	1.09
		C	21%	23%				
ATG5	rs3804333	C	79%	79%	40/40	0.9691	0.8769	1.02
		T	21%	21%				
ATG5	rs490010	G	53%	50%	37/37	0.7150	0.8410	1.13
		A	47%	50%				
ATG16L1	rs6752107	A	58%	45%	40/40	0.0999	0.1370	1.69
		G	42%	55%				
ATG16L2	rs10751215	C	64%	46%	40/40	0.0240	0.0359	2.07
		T	36%	54%				
IRGM	rs10059011	C	51%	45%	38/38	0.4396	0.5415	1.29
		A	49%	55%				

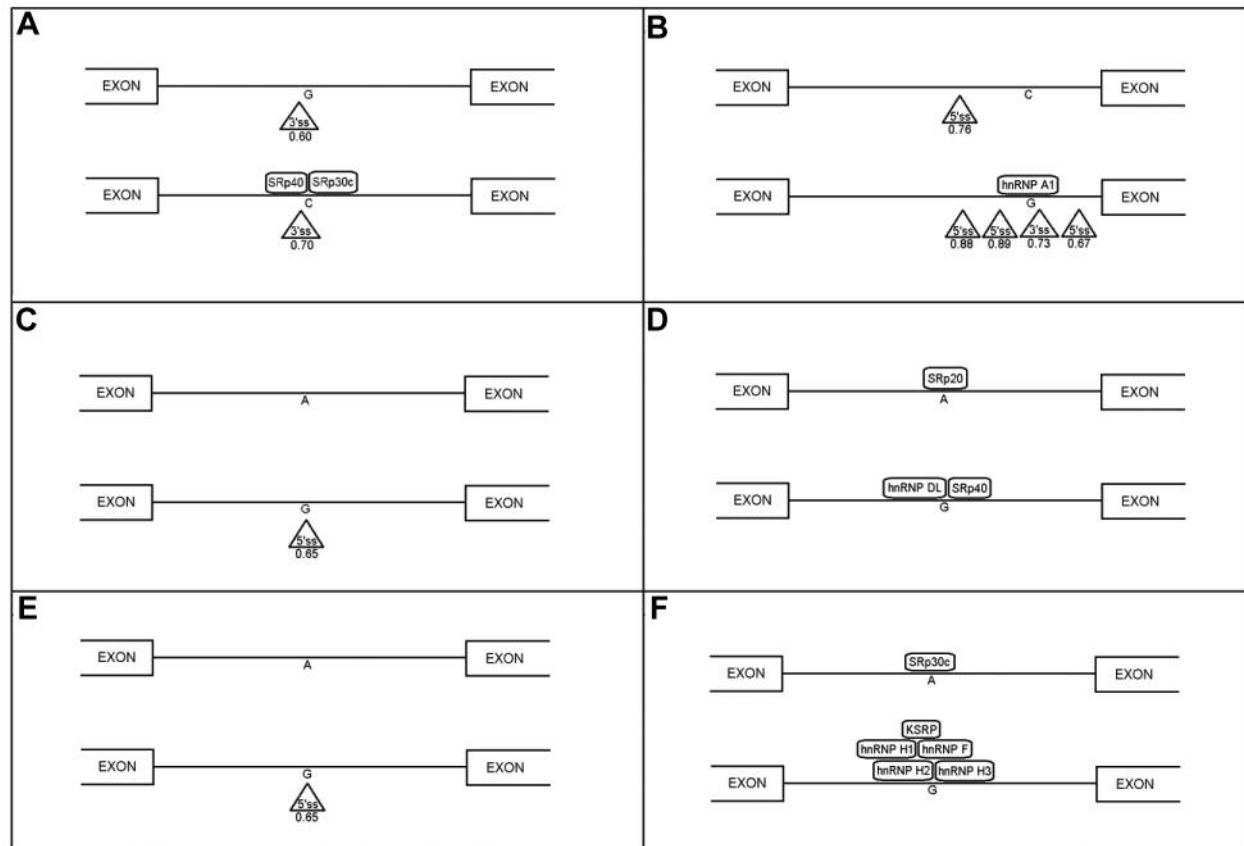


Figure 4. The identified *ATG4A* (A), *ATG4B* (B), *ATG4C* (C-E) and *ATG5* (F) single nucleotide polymorphisms (SNPs) and their predicted action on transcription regulation and splicing process.

rs6683832 of the *ATG4C* autophagy related 4C, cysteine peptidase gene weakens the cryptic 5'ss (AACTTGgttctgtc) according to HSF so no splicing alterations are expected (Figure 4E).

rs9373839 of the *ATG5* autophagy related 5 gene creates a binding site for KSRP, hnRNP F, hnRNP H1, hnRNP H2 and hnRNP H3 splicing proteins to the detriment of the SRp30c exonic enhancer. The binding of these proteins could reinforce the intron definition so no splicing effects are predicted (Figure 4F).

rs3804333 of the *ATG5* autophagy related 5 gene allows the binding of the hnRNP A1 exonic silencer instead of SRp30c and SF2/ASF exonic enhancers without effects (Figure 5A).

rs490010 of the *ATG5* autophagy related 5 gene prevents the binding of SRp20 and YB-1 splicing factors but this should not alter the Intron removal (Figure 5B).

rs6752107 of the *ATG16L1* autophagy related 16-like 1 gene creates a weak cryptic 5'ss (cttaaggGTACAAAA)

according to NNSPLICE and destroys a cryptic 3'ss (cttaagagTACAAA) according to (HSF). Moreover, this polymorphism reinforces another cryptic 5'ss (TTAAGGgtacaaaa) raising its score to 0.70. Although these changes could provoke alterations, these should be avoided by the predicted binding of SF2/ASF, hnRNP F, hnRNP C2, hnRNP H1, hnRNP H2 and hnRNP H3 proteins (Figure 5C).

rs10751215 of the *ATG16L2* autophagy related 16-like 2 gene could cause the loss of SC35 and SF2/ASF splicing factors and the binding of SF1 and MBNL1 proteins. Since SpliceAid tool has predicted that in this tract there could be many other binding sites for SC35, SF2/ASF, SF1 and MBNL1 no splicing alterations are expected (Figure 5D).

rs10059011 of the *IRGM* immunity-related GTPase family, M gene lies within the first non-coding exon (5'UTR) but no difference in terms of splicing sites or protein binding was predicted (Figure 5E).

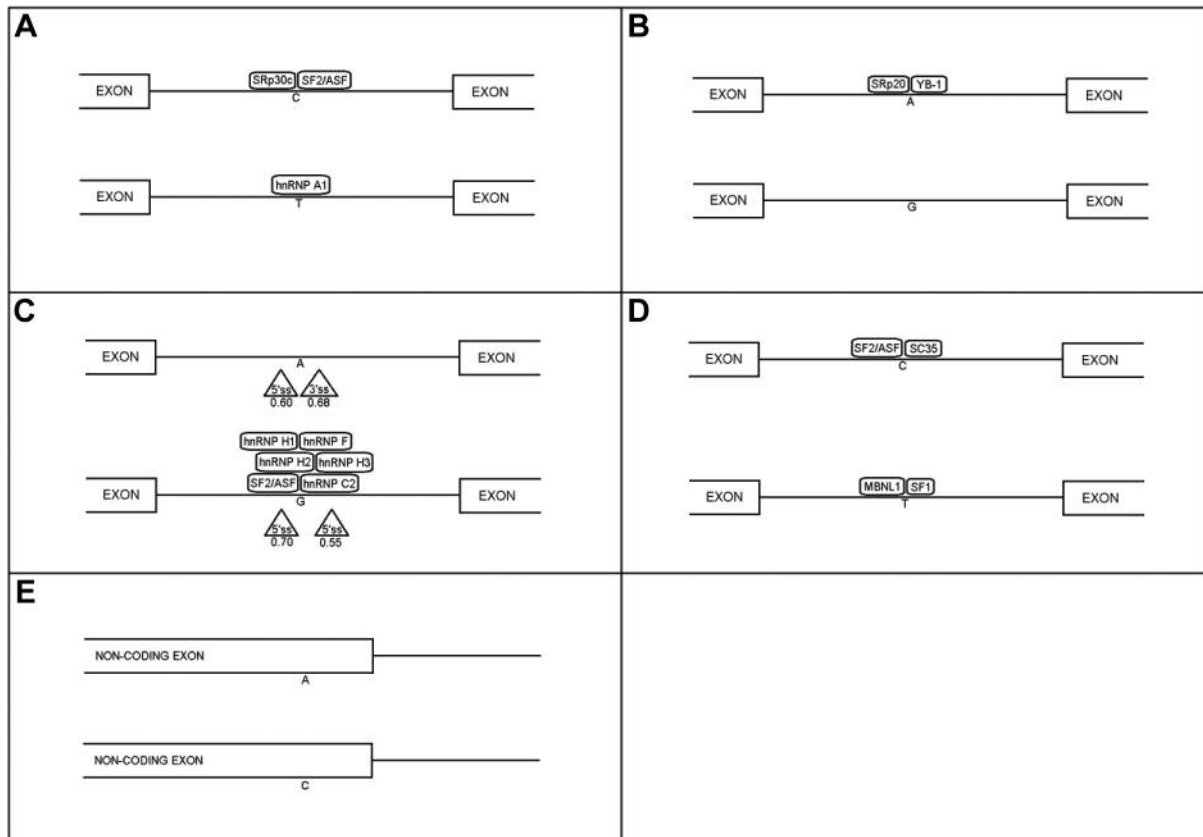


Figure 5. The identified *ATG5* (A-B), *ATG16L1* (C), *ATG16L2* (D) and *IRGM* (E) single nucleotide polymorphisms (SNPs) and their predicted action on transcription regulation and splicing process.

Discussion

Several targeted agents are now available for the treatment of mRCC, but the lack of validated predictive and prognostic factors for the selection of patients who may benefit from pazopanib, rather than sunitinib, not only limits the possibility of optimizing the efficacy and tolerability of these drugs but also does not allow the design of personalized combined or sequential strategies for RCC patients. The transition from “one size fits all” to “a molecularly tailored” or “personalized” approach for advanced RCC patients represents a fundamental goal for the current translational studies.

In the last decade, SNPs have emerged as potential predictive and prognostic factors in patients with RCC treated with TKIs (27-33). Xu *et al.* observed that 3 polymorphisms in *IL8* and *HIF1A* and 5 polymorphisms in *HIF1A*, *NR1I2*, and *VEGFA* were significantly correlated with the PFS and response rate (RR) of patients treated with pazopanib (27). In addition, they showed that genetic variants of *IL-8* associated with higher gene expression conferred the ability to escape faster from the VEGFR

blockade, resulting in poorer survival outcomes in pazopanib- or sunitinib-treated RCC patients (31).

In 2013, we revealed that pazopanib was able to induce autophagic cell death in RCC cell lines (13). Based on this evidence, we first tried to evaluate in this study the potential prognostic role of autophagic gene polymorphisms in patients treated with first-line pazopanib for metastatic disease. We showed that *ATG4*rs7880351, *ATG4C* rs6670694, *ATG4C* rs6683832 and *ATG5* rs490010 polymorphisms were correlated with the PFS of patients treated with pazopanib. Moreover, the presence of *ATG16L2* polymorphism rs10751215 was significantly associated with a lower risk of developing clear cell RCC compared to the general population.

Furthermore, we reported the predicted molecular effects of some polymorphisms related to autophagy. Although no severe effects have been predicted, the preservation of the splicing patterns of these genes should be experimentally assessed in the renal tissue. In addition, since the splicing process is very specific, interesting differences could be found between healthy and pathologic tissues.

Our study presents several limitations, including its retrospective nature, which is susceptible to bias in data selection and analysis, and the small number of evaluated patients. However, to the best of our knowledge, there are no studies focusing on the correlation between the referred SNPs and RCC risk and prognosis.

Conclusion

Our results suggest, for the first time, that inherited abnormalities in *ATG4* and *ATG5* genes influence the risk and response to treatment of RCC. However, we recognize that our findings will require confirmation in larger perspective epidemiological and clinical studies focusing on the prognostic significance of SNPs in patients with metastatic RCC.

Conflicts of Interest

The Authors declare to have no conflicts of interest.

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