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Research Article

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Low *ASH1L* Expression as Potential Diagnostic and Prognostic Biomarker for Renal Cell Carcinoma

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Abstract

As an important methyltransferase, ASH1L played main roles in cell differentiation, embryonic development and auto-immune response. It had been reported that its abnormal expression was closely related to the progression of some diseases. In the current study, we found that ASH1L was low expressed in renal cell carcinoma, and its low expression was positively correlated with tumor progression. Patients with low ASH1L expression had poor OS and RFS, and it had excellent clinical diagnostic value. Furthermore, lower ASH1L expression in dead than survival patients, and multivariate regression Cox analysis confirmed that low ASH1L expression was a predictor for poor prognosis of patients with renal cell carcinoma. Gene-set-enrichment-analysis showed that the DNA-repair, reactive-oxygen-species pathway and Myc-target V2 signaling were significantly enriched to the low ASH1L expression phenotype. Taking together, our findings demonstrated that the low ASH1L expression was likely to be useful as a promising prognostic indicator for renal cell carcinoma.

Keywords: Renal Cell Carcinoma, Histone Methylation, *ASH1L*, Poor Prognosis, Biomarker

Introduction

Renal cell carcinoma was the disease with the highest fatality rate (approximately 25%) among malignant tumors of the urinary system [1-4]. The incidence of renal cell carcinoma was gradually steadily increasing in the most countries, including China and American [5, 6]. Although significant improvements had been made in treatment, the prognosis of patients remained frustrating due to local recurrence and organ metastasis [7]. The main reason was that treatment targets and follow-up biomarkers are limited. Thus, the screening of new molecular targets and biomarkers was helpful to the treatment selection and prognosis assessment of patients.

Methylation, an important epigenetic modification system, was a regulator of gene expression. It mainly included DNA methylation and histone methylation [8, 9]. This system was closely related to diseases such as senescence, Alzheimer's and cancer [10-12]. Recently, genome-wide studies had determined that histones and chromatin modulators were one of the common dysfunction categories in some cancer types [13]. These findings provided many potential therapeutic targets in tumor treatment options, including many molecules that regulated histone methylation, such as lysine-specific demethylase 1 and *ASH1L* [14].

Absent, small, or homeotic 1-like (*ASH1L*), originally found in Drosophila, is a lysine methyltransferase of histone. In mammals, it can specifically activate the methylation of specific histones H3K4 and H3K36 to regulate gene expression, and it is involved in

the processes of cell differentiation, embryonic development and auto-immune response [15-17]. The histone H3K36 catalyzed by *ASH1L* caused the activation of the leukemia driver HOX, leading to the onset of the disease [18]. Recent studies had shown that *ASH1L* was widely expressed in a variety of tumors, including liver cancer, breast cancer and thyroid cancer [19-21]. Importantly, its expression was associated with poor survival in breast cancer patients [20]. These findings indicated that *ASH1L* had major roles in tumor onset and progression. However, the roles of its expression in clinical diagnosis and prognostic monitoring are rarely reported.

In this study, we revealed the expression patterns of *ASH1L* in different stages of KIRC, and analyzed the diagnostic value of its expression. In addition, we also suggested the effect of *ASH1L* expression on different clinical characteristics of patients, including the overall survival (OS) and recurrence-free survival (RFS). Our results clarified that *ASH1L* was an independent predictor of poor prognosis in KIRC, which supported its clinical application as a biomarker for diagnosis and prognostic assessment.

Materials and Methods

Data-sets collecting of TCGA database

The RNASeq data of *ASH1L* and clinical information of 538 patients were obtained from the TCGA-KIRC database by using the RTCGA-Tool-box packages in R software, including RTCGA.mRNA, RTCGA.rnaseq and RTCGA.clinical. All data-sets were processed in R software (version 4.0.1).

Gene-Sets Enrichment Analysis

The data of RTCGA.rnaseq was analyzed by selecting GSEA software (version 4.0.3), and the phenotypic difference was determined by "h.all.v7.2.symbols.gmt" gene-sets. $P<0.05$ and false-discovery-rate (FDR<0.25) are considered to be significantly enriched.

Data Analysis

The box plot showed the difference patterns in *ASH1L* expression, and the chi-square test assessed the relationship between its expression and the clinical characteristics of patients with KIRC. The pROC package in R software was used to evaluate the significance of *ASH1L* expression in the diagnosis of KIRC, and patients were divided into two groups (high *ASH1L* expression and low *ASH1L* expression) according to the ROC threshold. The ggsurvplot package was used to evaluate the effect of *ASH1L* expression on the overall survival (OS) of patients, as well as relapse-free survival (RFS). Cox regression model was selected to evaluate the risk prediction of *ASH1L* expression on poor prognosis. Statistical difference was recorded as P value <0.5.

Results

The Clinical Features of Study Population

The pathological process and health status of patients can be reflected by their clinical characteristics [22]. We obtained clinical data-sets of 538 patients from TCGA-KIRC, mainly including age, sex and histological grade, as well as pathologic stage, MNT classifications and survival status (Table 1).

Table 1: The clinical characteristics of patients in KIRC

Parameters	Numbers (%)
Age	
≥55	374 (69.52)
<55	164 (30.48)
Genger	
Male	347 (64.50)
Female	191(35.50)
Histologic grade	
NA	3(0.56)
G1	14(2.60)
G2	230 (42.75)
G3	208 (38.66)
G4	78 (14.50)
GX	5(0.93)
Pathologic stage	
I	271 (50.37)
II	57 (10.59)
III	126(23.42)
IV	84 (15.61)
T classification	
T1	276(51.30)

T2	69 (12.83)
T3	182 (33.83)
T4	11 (2.04)
N classification	
N0	240 (44.61)
N1	17 (3.16)
NX	281(52.23)
M classification	
NA	2(0.37)
M0	426 (79.18)
M1	79 (14.68)
MX	31(5.76)
Vital status	
Dead	162 (30.11)
Survival	376 (69.89)
Relapse	
NA	28(5.20)
NO	364(67.66)
YES	146(27.14)
ASH1L expression	
NA	4(0.74)
High	187(34.76)
Low	347(64.50)

Note: NA (Not available)

The expression patterns of *ASH1L* among patients with KIRC

To determine the effect of ASH1L expression on the different clinical characteristics of patients with KIRC, we compared its expression in normal and diseased tissues. We found that *ASH1L* expression was significantly reduced (P <0.0001; Figure 1). In addition, the box-plot showed that its expression was observed to be significant difference according to histologic grade (P <0.0001), pathologic stage (P <0.0001), T classification (P =0.00017), M classification (P =0.0018) and vital status (P<0.0001). These results indicate that *ASH1L* expression is down-regulated in KIRC and is related to disease deterioration and survival status.

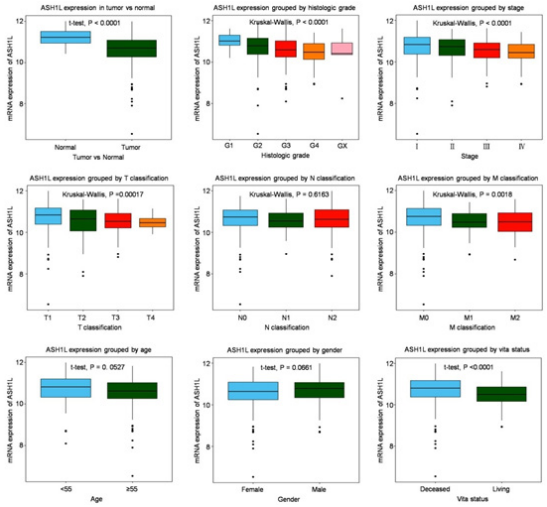

Figure 1: The expression of *ASH1L* in different clinical characteristics of KIRC patients. The expression patterns of *ASH1L* was compared in different sub-groups, including normal vs tumor, patient's histologic grade, pathologic stage, T/N/M classification, age, gender and vital status.

The Potential Relationship Between *ASH1L* Expression and Clinical Characteristics in KIRC

We previously found that *ASH1L* expression was related to the progression of KIRC. Next, we chose the chi-square test to evaluate the potential correlation between its expression and the patient's clinic-pathological features. Our results suggested that low *ASH1L* expression was significantly correlated with histologic grade (P = 0.004), pathologic stage (P = 0.001), M classification (P = 0.018) and T classification (P = 0.001), and negatively correlated with survival status.

Table 2: The collection between the clinicopathologic features and *ASH1L* expression.

Parameters	Variables	Numbers	*ASH1L*				X2	P-value
			high	Prop (%)	low	Prop(%)		
age	≥55	372	122	65.24	250	72.05	2.663	0.103
	<55	162	65	34.76	97	72.95		
Genger	Male	346	116	62.03	230	66.28	0.962	0.327
	Female	188	71	37.97	117	33.72		
Histologic grade	G1	14	8	4.30	6	1.74	15.552	0.004
	G2	229	97	52.15	132	38.26		
	G3	207	62	33.33	145	42.03		
	G4	76	18	9.68	58	16.81		
	GX	5	1	0.54	4	1.16		
Pathologic stage	I	269	117	62.57	152	43.80	21.861	0.001
	II	57	22	11.76	35	10.09		
	III	124	29	15.51	95	27.38		
	IV	84	19	10.16	65	18.73		
M classification	M0	422	161	86.10	261	75.65	8.086	0.018
	M1	79	19	10.16	60	17.39		
	MX	31	7	3.74	24	6.96		
N classification	N0	240	88	47.06	152	43.80	1.080	0.583
	N1	16	4	2.14	12	3.46		
	NX	278	95	50.80	183	52.74		
T classification	T1	274	119	63.64	155	44.67	22.616	0.001
	T2	69	25	13.37	44	12.68		
	T3	180	42	22.46	138	39.77		
	T4	11	1	0.53	10	2.88		
Vital status	Dead	160	34	18.18	126	36.31	19.032	0.001
	Survival	374	153	81.82	221	63.69		

The Diagnostic Value of *ASH1L* Expression in KIRC

In order to analyze the effect of *ASH1L* expression on the diagnostic value of KIRC, the pROC package in R software was established. We found that *ASH1L* had excellent clinical diagnostic value (AUC=0.790; Figure 2A). Subsequently, we also revealed the diagnostic value of different stages of tumors, including stage I (AUC = 0.743), stage II (AUC = 0.785), stage III (AUC = 0.842) and stage IV (AUC = 0.871).

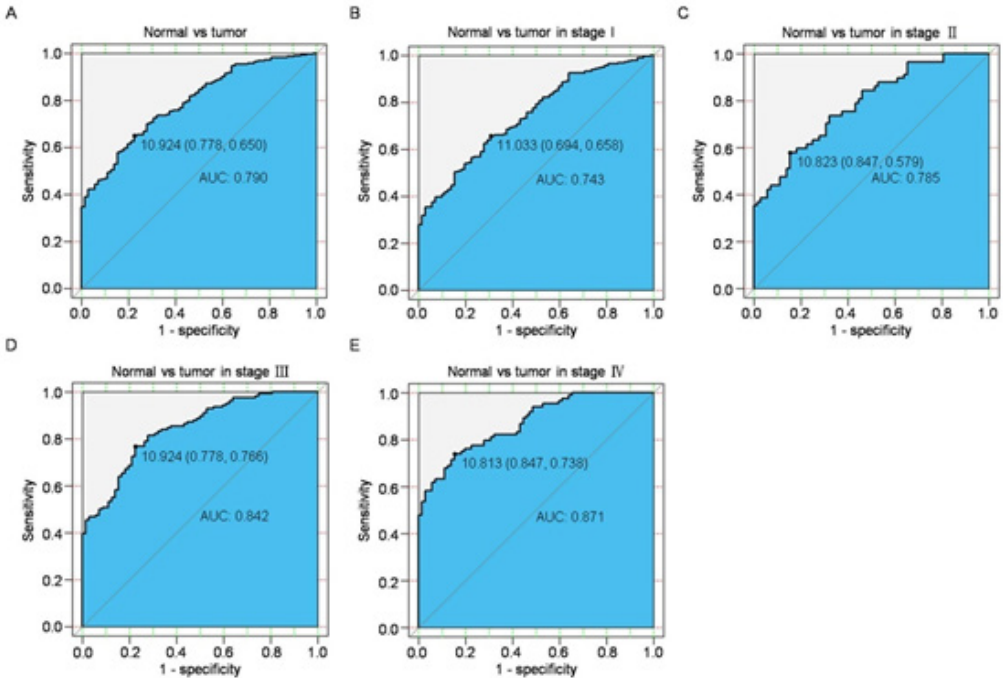

Figure 2: The clinical diagnosis value of *ASH1L* expression in KIRC. ROC curves was performed to evaluate the diagnosis value of *ASH1L* expression in cancerous vs. normal tissues, as well as in different pathologic stages.

Low *ASH1L* Expression is a Poor Prognostic Factor for OS Among KIRC Patients

In order to examine the effect of *ASH1L* expression on OS of KIRC patients, Kaplan Meier survival curves were selected and implemented. As shown in Figure 3, patients with low *ASH1L* expression had a shorter OS ($P < 0.0001$), including female patients ($P = 0.0054$) and male patients ($P = 0.0029$). In addition, its low expression was also significantly related to OS of patients in G1/G2 ($P = 0.034$), G3/G4/GX ($P = 0.0075$), stage III/IV ($P = 0.012$). T1 ($P = 0.0093$), N0 ($P = 0.027$), N1/NX ($P = 0.00055$) and M0 ($P = 0.00028$). Next, the univariate and multivariate analysis were performed through risk regression model. As shown in Table 3, low *ASH1L* expression was a poor prognostic factor for OS in KIRC patients (hazard ratio = 1.56, 95% confidence interval: 1.06–2.30, $P = 0.025$).

Table 3: Univariate and Multivariate analysis of Over Survival in KIRC patients.

	Univariate analysis			Multivariate analysis		
	Hazard Ratio	**CI95**	**Pvalue**	**Hazard Ratio**	**CI95**	**Pvalue**
Age	1.928	1.314-2.828	0.001	1.568	1.055-2.330	0.026
Gender	1.042	0.757-1.435	0.799			
Histologic grade	2.057	1.713-2.471	0.001	1.515	1.220-1.880	0.001
Pathologic stage	1.962	1.712-2.251	0.001	2.084	1.429-3.039	0.001
M classification	2.328	1.824-2.971	0.001	0.848	0.514-1.400	0.982
N classification	0.858	0.734-1.005	0.057			
T classification	2.073	1.746-2.461	0.001	0.772	0.590-1.133	0.260
ASH1L	2.156	1.476-3.151	0.001	1.560	1.058-2.300	0.025

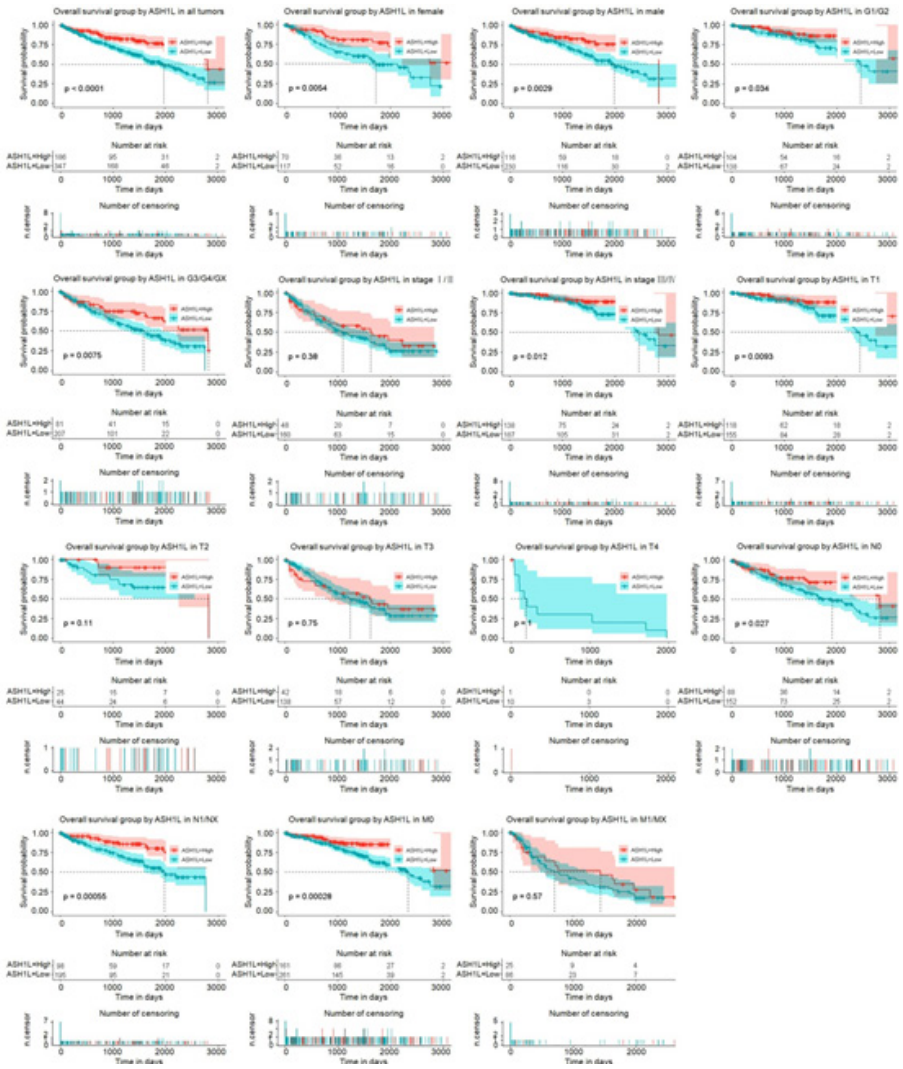

Figure 3: Effect of *ASH1L* expression on OS in KIRC. Survival curves of *ASH1L* expression in all patients and in different subgroup.

Low *ASH1L* expression is a poor prognostic factor for RFS among KIRC patients

We continued to construct survival curve to explore the effect of *ASH1L* expression on RFS of KIRC patients, and found that patients with low *ASH1L* expression had a shorter RFS (P < 0.0001; figure 4). Additionally, we also found that both female patients (P = 0.00370) and male (P = 0.0310) with low *ASH1L* expression had a shorter RFS. Its low expression was also significantly related to RFS of patients in G1/G2 (P = 0.039), G3/G4/GX (P = 0.0063), N0 (P = 0.045), N1/NX (P = 0.00016) and M0 (P = 0.00082). Subsequently, we used risk regression model to perform the univariate and multivariate analysis. As shown in Table 4, low *ASH1L* expression was a poor prognostic factor for RFS in KIRC patients (hazard ratio = 1.67, 95% confidence interval: 1.11–2.52, P = 0.014).

Table 4: Univariate and Multivariate analysis of Relapse-Free Survival in KIRC patients.

	Univariate analysis			Multivariate analysis		
	Hazard Ratio	CI95	Pvalue	Hazard Ratio	CI95	Pvalue
Age	1.370	0.951-1.973	0.091			
Gender	0.769	0.539-1.097	0.147			
Histologic grade	1.968	1.625-2.382	0.001	1.271	1.018-1.587	0.001
Pathologic stage	2.393	2.049-2.795	0.001	2.520	1.755-3.617	0.001
M classification	3.198	2.537-4.032	0.001	1.259	0.781-2.032	0.345

N classification	1.033	0.875-1.221	0.699			
T classification	2.319	1.923-2.797	0.001	0.713	0.492-1.034	0.491
ASH1L	2.263	1.519-3.371	0.001	1.671	1.110-2.515	0.014

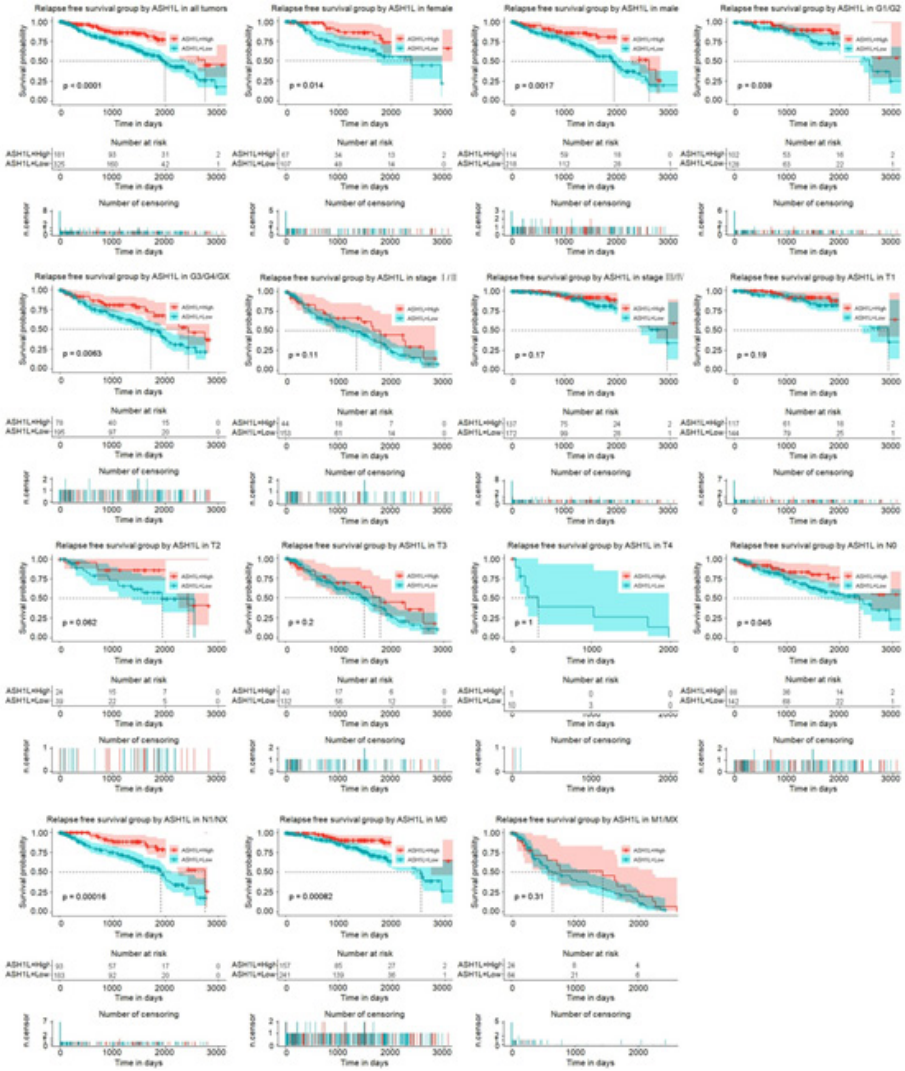

Figure 4: Effect of *ASH1L* expression on RFS in KIRC. Survival curves of *ASH1L* expression in all patients and in different subgroup.

The Gene-Set Enrichment Analysis in Low *ASH1L* Expression

We previously showed that low *ASH1L* expression was positively correlated with poor prognosis of KIRC. Subsequently, we used GSEA software to analyze the low and high *ASH1L* expression data-sets to determine the signal pathways activated in KIRC. The results showed that the DNA-repair, reactive-oxygen-species pathway and Myc-target V2 signaling were significantly enriched to the low *ASH1L* expression phenotype (Table 5, Figure 5).

Table 5: Gene-set-enrichment-analysis in low *ASH1L* expression phenotype in KIRC.

Name	ES	NES	NOM p-value
HALLMARK_DNA_REPAIR	0.53	1.89	0.002
HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY	0.54	1.73	0.002
HALLMARK_MYC_TARGETS_V2	0.61	1.70	0.026

ES: Enrichment score; NES: normalized enrichment score; NOM: nominal. GSEA with NOM P-value <0.05 was considered as significantly enriched.

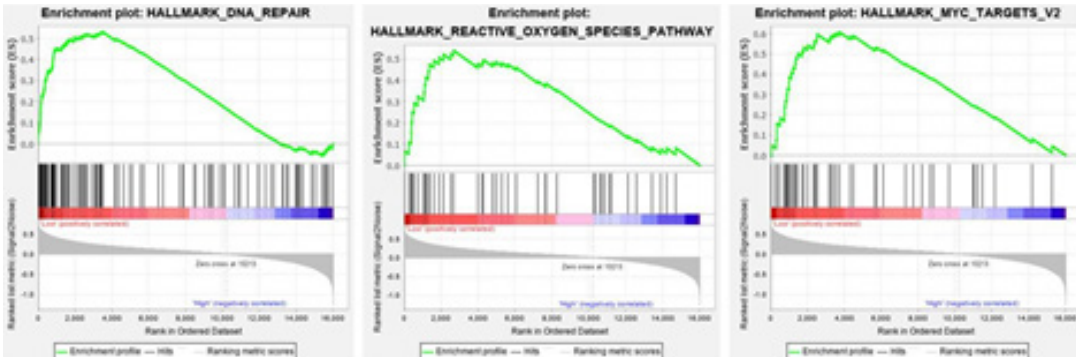

Figure 5: Gene-Set Enrichment analysis. GSEA results showing differential enrichment of genes related to the DNA repair, reactive oxygen species pathway and Myc-targets V2 in KIRC cases with low *ASH1L* expression.

Discussion

Here, we found that *ASH1L* expression was significantly reduced in the disease through the TCGA-KIRC clinical data-set, and its expression was related to survival status and tumor progression of patients. Furthermore, we suggested that *ASH1L* had excellent clinical diagnostic value, and its low expression was a risk reason for OS and RFS. Our multivariate regressions analysis indicated that low *ASH1L* expression was a potential biomarker in the clinical diagnosis and prognostic evaluation for KIRC.

As an important histone methyltransferase, *ASH1L* had important functions in cell proliferation, immune response and transcriptional regulation [23-25]. Recently, studies had found that the abnormal expression of *ASH1L* was related to the occurrence of leukemia [26]. It had been shown to be over-expressed in hepatocellular carcinoma and breast cancer [22, 24]. It meant that *ASH1L* played important roles in tumor onset and progression, although whether it exerted biology-roles in these diseases was uncertain. Unlike previous findings, however, we suggested low *ASH1L* expression in KIRC [27, 28]. It indicated that *ASH1L* expression had the specificity of cells or tissues. Its expression gradually decreased as the increase of histologic grade, stage, T and M classifications, which indicated that *ASH1L* was closely related to proliferation, migration, and invasion of tumor cells.

In the immune response, *ASH1L* inhibited Interleukin-6 (IL-6) expression by activating NF-kB and Mitogen-activated protein kinase (MAPK) signaling pathways, thereby preventing toxin-shock and auto-immune diseases in vivo [29]. *ASH1L* regulated the hand-off between damage recognition factors in global-genome nucleotide excision repair [30]. In addition, *ASH1L* together with miR-375 played critical roles in human hepatocellular carcinoma as a tumor suppressor [31]. In this study, we revealed the signaling pathways that are significantly enriched in the low *ASH1L* expression phenotype, including DNA-repair, reactive-oxygen-species pathway and Myc-target V2 signaling. It indicated that *ASH1L* might regulate tumor progression through the above-mentioned signaling pathway. Moreover, we found that *ASH1L* had excellent clinical diagnostic value and was able to also diagnose KIRC patients at different stages.

Considering the important roles of prognostic monitoring in the clinical treatment of tumors, indeed, the regular follow-up of molecular markers will help to formulate accurate projects for diagnosis and treatment [32]. It had been reported that the expression of *ASH1L* was related to the prognosis of breast cancer patients [33]. This again demonstrated the potential contribution of *ASH1L* expression in KIRC treatment options. In this study, patients with low *ASH1L* expression had poor OS and RFS. In addition, we revealed that lower *ASH1L* expression in dead than survival patients, suggesting it was likely to be useful as a prognostic indicator.

To our knowledge, this study is the first to identify the effect of *ASH1L* expression on different clinical characteristics of patients by collecting TCGA-KIRC clinical data-sets. We provided evidence of *ASH1L* expression in the diagnosis and prognosis, and it was an independent predictor of poor prognosis. However, subject to sample size restrictions, it is difficult to establish a more valuable predictive model between *ASH1L* expression and the clinic pathological data of KIRC patients. We need to expand the sample size in order to build a better prediction model in our follow-up research.

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