ORIGINAL RESEARCH

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RIPK3 expression as a potential predictive and prognostic marker in metastatic colon cancer

Abstract

Background: Colorectal cancer is one of the primary causes of cancer-related deaths and 5-fluorouracil (5-FU) therapy remains the cornerstone of treatment in these patients. Resistance to 5-FU represents a major obstacle; therefore, finding new predictive and prognostic markers is crucial for improvement of patient outcomes. Recently a new type of programmed cell death was discovered—necroptosis, which depends on receptor interacting protein 3 (RIPK3). Preclinical data showed that necroptotic cell death is an important effector mechanism of 5-FU-mediated anticancer activity.

Purpose: To investigate the predictive and prognostic performance of RIPK3 expression in primary tumors.

Methods: Colon cancer patients (n=74) with metastatic stage were included in this retrospective study and all were treated with first-line 5-FU based chemotherapy. Immunohistochemical staining was performed.

Results: The progression free survival for the low expression group of RIPK3 was 5.6 months (95% CI, 4.4-6.8) vs 8.4 months (95% CI, 6.4-10.3) of the group with high expression (p=0.02). Moreover, patients with high expression of RIPK3 were associated with lower risk of disease progression HR 0.61 (95% CI, 0.38-0.97; p=0.044). Patients with high expression levels of RIPK3 also had significantly longer mean overall survival (OS) of 29.3 months (95% CI, 20.8-37.8) as compared with those with low expression: 18.5 months (95% CI, 15.06-21.9) (p= 0.036). In addition, univariate analysis showed that high level of RIPK3 expression was associated with a longer OS HR 0.59 (95% CI, 0.35-0.98; p=0.044).

Conclusions: This study suggests that expression of RIPK3 in primary tumors of metastatic colon cancer patients should be further investigated for its potential as a promising predictive and prognostic marker.

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Colorectal cancer (CRC) is one of the leading causes of morbidity and cancer-related death worldwide [1]. The 5-year survival rate for patients with CRC has risen over the past several decades and now approaches 60%. Despite the fact that CRC can be prevented or cured if diagnosed early, approximately 25% of patients present with synchronous metastases at the time of diagnosis and almost 50% of patients are expected to develop metastases, resulting in high mortality rates [2]. Most of the chemotherapy regimens are 5-fluorouracil (5-FU) -based. Resistance towards 5-FU is a key challenge in the treatment of these patients; therefore, finding reliable, predictive and prognostic markers is crucial for the improvement of patient outcomes.

Recently, a new type of programmed cell death called "necroptosis" has been discovered [3,4]. Similar to apoptosis, this process is tightly regulated by distinct molecules and has morphological features of both necrosis and inflammation. Nevertheless, the exact pathways activating this programmed necrosis are not yet fully understood. It has been shown that tumor necrosis factor alpha (TNF- α) is the trigger factor for the formation of a complex (necrosome) in the cytoplasm between receptor-interacting protein kinase 1 (RIPK1) and receptor-interacting protein kinase 3 (RIPK3) [5,6]. In the formation process of this complex, RIPK3 phosphorylates RIPK1, which is an essential step for inducing necroptosis [7]. In vitro and in vivo data have shown that necroptotic cell death is an important effector mechanism of 5-FU-mediated anti-tumoral activity [8,9].

The aim of our retrospective study was to evaluate the predictive and prognostic performance of RIPK3 expression in primary tumors in 74 patients with metastatic colon cancer.

Methods

Patient selection

In this retrospective study we reviewed the cases of 74 patients with metastatic colon cancer who were treated with first line 5-FU based regimen between January 1, 2012 and December 31, 2015 at UMHAT St. Marina, Varna, Bulgaria. Available archival tissue samples from the 74 patients were collected. The procedure was approved by the Scientific Research Ethics Committee at the Prof. Dr. Paraskev Stoyanov Medical University in Varna. The tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors (ver.1.1) (RECIST 1.1). Patients were staged before treatment by performing computed tomography (CT) or positron emission tomography. The patient data, which

included demographic information, radiological test results, disease characteristics and therapy regimens were retrospectively obtained from the department's archive of patients' files.

Immunohistochemical staining

Specimens of primary colon adenocarcinoma were obtained from 74 patients at the Clinical Pathology Department at St. Marina University Hospital. The diagnosis was microscopically confirmed by a pathologist. All hematoxylin-eosin-stained specimens contained cancerous tissues. Five-micrometer sections were cut from the paraffin blocks and placed on glass slides. Sections were deparaffinized with xylene and dehydrated in a graded series of ethanol to DI water. Antigen retrieval was performed in the pre-heated EnVision FLEX Target Retrieval Solution (working solution) in PT Link tanks and incubated for 30 minutes at 97°C at pH=9. After cooling, the slides were placed in a diluted, at room temperature FLEX Wash Buffer (20x) for 1-5 minutes. Sections were stained by using FLEX protocol in Dako Autostainer/Autostainer Plus. Samples were tested with recombinant rabbit polyclonal antibody to RIPK3 (ABCAM's RabMab technology ab56164). The antibody (Anti- RIPK3, diluted 1:100) was incubated for 20 minutes. Detection of expression levels of RIPK3 was achieved by using the UltraVision detection system Anti-polyvalent, HRP/DAB. Finally, the reaction was visualized by the appropriate substrate-chromogen (DAB, Diaminobenzidine) reagent. Counterstaining was done using Mayer's haematoxylin for the evaluation of immunostaining. Digital images were obtained using the Leica Aperio ScanScope AT2 device (Aperio Technologies, Vista, CA) and further analyses of the scanned images were performed with the V12.1.0.5029 (Aperio) ImageScope.

H-score assessment

Two independent pathologists, with no prior knowledge of the clinical data, scored all immunohistochemically-stained specimens of RIPK3, based on the staining intensity and the percentage of positively stained tumor cells. The H-score was defined as the percentage of cells with weak stain intensity, plus two times the percentage of cells with moderate stain intensity, plus three times the percentage of cells with strong stain intensity [10]. The median value of these was used to group cases into two categories of low ($\leq$ median; 62.5 ± 35.8) and high ($>$ median; 209.1 ± 44.1) expression (Figure 1).

FIGURE 1. Immunohistochemical staining of RIPK3 in human colon carcinomas

A: patient with low RIPK3 expression; B: patient with high RIPK3 expression. (20x magnification)

KRAS Mutational Analysis

Tissue sections were examined under microscope using hematoxylin and eosin staining. The samples were then manually macrodissected and DNA was extracted from paraffin-embedded formalin-fixed tumor tissue and Kirsten rat sarcoma viral oncogene homolog (KRAS) mutational status was determined by allele-specific real-time PCR- based assay (AMOY Dx KRAS Seven Mutations Detection Kit,

Amoy Diagnostics Co., Ltd., China) in the National Genetic laboratory in Sofia, Bulgaria.

Endpoints

Clinical benefit rate (CBR) was defined as the proportion of patients with a partial response or stable disease for at least six months. No patients with complete response were recorded. The clinical tumor response was assessed by CT or positron emission tomography every three months, according to the Response Evaluation Criteria in Solid Tumors (ver.1.1) [11]. Progression free survival (PFS) was defined as the time elapsed between treatment initiation and tumor progression or death from any cause. Overall survival (OS) was defined as the interval between diagnosis of the disease and death or date of last follow-up evaluation.

Statistical design and analysis

Data were managed and analyzed using SPSS software ver. 23. The Mann-Whitney U test, χ^2 test and Spearman correlation were used to compare and evaluate the correlations between the expression of RIPK3 in primary tumors and the clinicopathological characteristics of the patients such as age, gender, KRAS status, histopathological grading, PS (ECOG)-performance status (Eastern Cooperative Oncology Group), CEA (carcinoembryonic antigen), number of metastatic sites and primary tumor location (left or right). Odds ratios (ORs) with confidence intervals (CIs) for categorical outcomes were calculated using a binary logistic regression model. Survival curves according to the RIPK3 expression in primary tumors were estimated using the Kaplan-Meier method, and differences were assessed using the log-rank test. Hazard ratios (HRs) and 95 percent confidence intervals for univariate and multivariate models were computed with the use of Cox proportional-hazards regression models. Two-tailed p-values (<0.05) were considered significant.

Results

Patient characteristics

In this retrospective study, we included 74 colon cancer patients with unresectable metastatic disease who received first line 5-FU-based chemotherapy. Each of the patients' performance status was assessed to be < 2 . The correlation between RIPK3 expression and clinicopathological characteristics is shown in Table 1. The mean age of the patients was 63.4 ± 10.5 years. The study population included

42 males (56.8%) and 32 females (43.2%). The number of patients with KRAS WT were n=48 (64.9%) and n=26 (35.1%) of the patients were with KRAS mutations. According to the histopathological classification 83.8% (n=62) were moderately differentiated (G2) and 16.2% (n=12) were poorly differentiated (G3). The most common metastatic sites were the liver (81.1%), lung (13.5%) and peritoneum (12.2%). Patients with high serum CEA>1 upper limit of normal (ULN) were n=54 (72.9%). We determined that 67.6% (n=50) of the primary tumors came from the left side of the colon and 32.4% (n=24) of the tumors came from the right side; 21.6% (n=16) of the patients had more than one metastatic site at the time of the diagnosis and 78.4% (n=58) had one metastatic site. Half of the patients were with an ECOG status 0.

Correlation between RIPK3 expression and clinicopathological parameters

All patients were treated with first-line chemotherapy (CT) in the form of FOLFOX (Leucovorin, 5-FU and Oxaliplatin), FOLFIRI (Leucovorin, 5-FU and Irinotecan) with or without Bevacizumab/Panitumumab, CAPEOX (Capecitabine + Oxaliplatin) and de Gramont (Leucovorin+5-FU) until progression of the disease. In total, 41 (55.4%) of the patients received FOLFOX4/CAPEOX ± Bevacizumab/Panitumumab, 22 (29.8%) received - FOLFIRI ± Bevacizumab/Panitumumab and 11 (14.8%) - de Gramont. The cycles were repeated every two weeks. The patients received at least 3 months of treatment. A total of 60 (81.1%) patients received full dose-intense chemotherapy, while 14 (18.9%) patients required modification of the dose or chemotherapy-free intervals.

No significant correlation was observed between the expression of RIPK3 and patients' age, gender, KRAS status, histological grade, primary tumor location, number of unresectable metastatic sites, PS (ECOG), types of regimens and CEA (Table 1).

Effects of RIPK3 expression on CBR, PFS and OS

The CBR (PR+SD for at least six months) was 35.1%. Patients with low level of RIPK3 expression showed significantly lower response rates than patients with high-level RIPK3 expression (n=6, 16.2% vs n=20, 54.1%). Moreover, considering chemotherapy response in terms of CBR, high expression of RIPK3 might be a positive predictive marker for response rate (OR 0.165, 95% CI

0.05-0.49, P = 0.01). No patients with complete response were recorded.

TABLE 1. Correlation between RIPK3 expression and clinicopathological characteristics

Clinicopathological characteristics	Low-level RIPK3 expression	High-level RIPK3 expression	P value ^a
Age (years)			
<65	19 (51.4)	18 (48.6)	0.8
≥65	18 (48.6)	19 (51.4)	
Sex			
Male	23 (54.8)	19 (45.2)	0.3
Female	14 (43.8)	18 (56.3)	
Histological grade			
Moderately	28 (45.2)	34 (54.8)	0.07
Poorly	9 (75.0)	3 (25.0)	
Metastatic site			
Liver			
Yes	29 (78.4)	31 (83.8)	0.5
No	8 (21.6)	6 (16.2)	
Peritoneum			
Yes	3 (8.1)	6 (16.2)	0.2
No	34 (91.9)	31 (83.8)	
Lung			
Yes	7 (18.9)	5 (13.5)	0.5
No	30 (81.1)	32 (86.5)	
Chemotherapy response			
SD (≥ 6 months)+ PR	6 (23.1)	20 (76.9)	0.001
PD	31 (64.6)	17 (35.4)	
KRAS			
M+	14 (53.8)	12 (46.2)	0.6
WT	23 (47.9)	25 (52.1)	

Primary tumor location			
Left colon	22 (44)	28 (56)	0.1
Right colon	15 (62.5)	9 (27.5)	
Number of unresectable metastatic sites			
1	29 (50)	29 (50)	1
>1	8 (50)	8 (50)	
PS (ECOG)			
0	15 (40.5)	22 (59.5)	0.1
1	22 (59.5)	15 (40.5)	
CEA			
≤1 ULN	11 (55)	9 (45)	0.6
>1 ULN	26 (48.1)	28 (51.9)	
Regimens			
FOLFOX4 / CAPEOX ± targeted therapy	21 (51.2)	20 (48.8)	0.8
FOLFIRI ± targeted therapy	10 (45.5)	12 (54.8)	
de Gramont	6 (54.5)	5 (45.5)	

a χ^2 test was used to compare and evaluate the correlations between the expression of RIPK3 in primary tumors and the patients' clinicopathological characteristics

Abbreviations: CEA, carcinoembryonic antigen; M+, mutation detected; PD, progressive disease; PR, partial response; PS (ECOG), performance status (Eastern Cooperative Oncology Group); RIPK3, receptor interacting protein 3; SD, stable disease; ULN, upper limit of normal; WT, wild type

Patients with high expression levels of RIPK3 in the primary tumor showed significantly longer mean PFS of 8.4 months (95% CI, 6.4-10.3) as compared with those with low expression levels of 5.6 months (95% CI, 4.4-6.8) (log rank test $p=0.02$) (Figure 2). Furthermore, in univariate analysis, high expression levels of RIPK3 in patients were associated with lower risk of disease progression HR 0.61 (95% CI, 0.38-0.97; $p=0.044$). In the multivariate analysis, high-level RIPK3 expression was an independent predictive factor for

longer PFS (HR 0.60, 95% CI 0.37-0.98, $P=0.045$) (Table 2).

Patients with high expression levels of RIPK3 also had significantly longer mean OS (29.3 months; 95% CI, 20.8-37.8) compared with those with low expression levels (18.5 months; 95% CI, 15.06-21.9) (log rank test $p=0.036$) (Figure 3). Also, in the univariate analysis, high level of RIPK3 expression and good performance status were associated with a longer OS HR (0.59; 95% CI, 0.35-0.98; $p=0.044$) and HR (0.55; 95% CI, 0.32-0.92; $p=0.02$), respectively. These associations were not apparent in the multivariate analysis (Table 3). As expected, differentiation of the tumor was an independent prognostic marker for survival.

FIGURE 2. Comparison of progression free survival based on RIPK3 expression levels

Kaplan-Meier estimates of progression free survival (PFS) according to RIPK3 expression in primary tumors of metastatic colon cancer patients (categorized as high and low compared with the median). There was a significant difference in the mean PFS between groups with high and low RIPK3 expression (low RIPK3 expression mean 5.6 months; 95% CI, 4.4 – 6.8 vs high RIPK3 expression mean 8.4 months; 95% CI, 6.4 – 10.3, log rank test $p=0.02$).

TABLE 2. Results of Cox regression analysis for predicting progression free survival

Variable	Univariate analysis			Multivariate analysis		
	H a z a r d ratio	95% CI	P value	H a z a r d ratio	95% CI	P value
Age	1.005	0.63-1.59	0.1	0.91	0.56-1.47	0.7
Gender	0.84	0.52-1.34	0.4	0.95	0.58-1.55	0.8
KRAS status	0.66	0.41-1.09	0.1	0.64	0.38-1.11	0.1
Histological grade	0.82	0.44–1.53	0.5	0.99	0.50-1.94	0.9
RIPK3 expression	0.61	0.38-0.97	0.044	0.60	0.37-0.98	0.045

TABLE 3. Results of Cox regression analysis for predicting overall survival

Variable	Univariate analysis			Multivariate analysis		
	H a z a r d ratio	95% CI	P value	H a z a r d ratio	95% CI	P value
Age	0.84	0.50-1.41	0.5	0.81	0.46-1.41	0.4
Gender	0.76	0.45-1.29	0.3	0.68	0.36-1.28	0.2
KRAS status	1.35	0.80-2.27	0.2	1.08	0.61-1.54	0.7
Histological grade	0.32	0.16-0.62	<0.001	0.34	0.16-0.79	0.003
RIPK3 expression	0.59	0.35-0.98	0.04	0.67	0.43-1.09	0.1
Primary tumor location	0.97	0.56-1.69	0.9	1.04	0.59-1.85	0.8
N u m b e r o f unresectable metastatic sites	1.37	0.75-2.5	0.3	1.89	0.97-3.69	0.06
PS (ECOG)	0.55	0.32-0.92	0.02	0.70	0.39-1.29	0.7
CEA	1.54	0.85-2.79	0.15	1.66	0.71-3.12	0.6

Discussion

The introduction of targeted agents has been shown to lead to a significant improvement in the progression-free and overall survival of patients with metastatic colon cancer [12]. Despite the advances in colorectal treatment and the increased knowledge of the relevant molecular pathways, our understanding of why some people benefit from treatment whereas others do not remains poor. Necroptosis is a caspase-independent type of cell death, which exhibits morphological characteristics, such as swelling of organelles

and plasma membrane rupture, and is involved in the pathogenesis of many human diseases including cancer [4,13].

Fluorouracil, an analog of uracil, is an anticancer prodrug that is converted intracellularly into three main, active metabolites. The beneficial effect of 5-FU-based chemotherapy has been linked to expression levels of several genes including thymidylate synthase (TS) and dihydropyrimidine dehydrogenase [14]. High levels of intratumoral expression of TS are a strong predictive marker

FIGURE 3. Comparison of overall survival based on RIPK3 expression levels

Kaplan-Meier estimates of overall survival (OS) according to RIPK3 expression in patients with metastatic colon cancer (categorized as high and low compared with the median). There was a significant difference in the mean OS between groups with high and low RIPK3 expression (low RIPK3 expression; mean 18.5 months; 95% CI, 15 – 21.9 vs high RIPK3 expression; mean 29.3 months; 95% CI, 20.8 – 37.9, log rank test $p=0.036$)

for poor response to 5-FU based chemotherapy regimens in CRC [15]. Fluorouracil resistance is a major clinical problem in the treatment of CRC [16]. Although 5-FU has been traditionally investigated for its potential to induce apoptosis, alternative forms of programmed cell death have been identified recently [17,18]. Similarly to apoptosis, necroptosis is mediated by cascade-like signalling pathways, but does not require the activation of caspases. Programmed necrosis mediated by RIPK3 has recently been characterized as a novel mechanism to induce cell death, with major functional importance in several organs, including the colon [4].

Cell culture experiments in human cell lines have demonstrated that the association of RIPK1 and RIPK3, as induced by TNF- α stimulation, serves as a key initial step in programmed necrosis [3]. Although the precise molecular mechanism of necroptosis remains unknown, it is believed that necroptosis is triggered when apoptosis is inactive and leads to rapid plasma membrane permeabilization, release of

intracellular contents and exposure of damage-associated molecular patterns [19]. Results from several studies support the idea that RIPK1–RIPK3-dependent necroptosis machinery may be a possible mechanism to limit tumor formation and progression [20].

Preclinical data with colon cancer cell lines have shown that administration of pan-caspase inhibitors blocks cleavage of RIPK3 and triggers necroptosis [20]. Treatment with 5-FU induces autocrine production of TNF- α , which shows that induction of necroptosis is an important effector mechanism of 5-FU-mediated anticancer activity [8]. In addition, in vivo xenograft experiments have demonstrated that a pan-caspase inhibitor, in combination with 5-FU, act synergistically to block tumor growth as compared with 5-FU alone. Notably, ex vivo experiments from this study showed that, when pretreated with pan-caspase inhibitor, more than 50% of fresh sliced tumor specimens from colorectal cancer patients produce more cell death by necroptosis in response to 5-FU. The RIPK3 expression prior to treatment was found to be crucial for the effect of conventional chemotherapeutics [21].

Others found that the expression levels of RIPK1 and RIPK3 were reduced in primary colon cancer tissues in comparison with normal adjacent tissues; this study, however, could not prove that these proteins play a major role in the chemotherapeutic agent-induced cell death of colon cancer cells [22].

Our results suggest that the expression level of RIPK3 in primary tumors might be a novel independent potential predictive biomarker of progression-free survival in patients with metastatic colon cancer. Furthermore, we found an association between expression of RIPK3, an essential marker of necroptosis, and CBR. High expression of RIPK3 was associated with a better OS, which makes RIPK3 a potential prognostic marker in patients with metastatic colon cancer disease.

Our study also indicated that high expression of RIPK3 in the primary tumor of patients was associated with a better survival prognosis in the univariate analysis, which suggests that necroptosis is an important determining factor of cancer cell death and outcome in patients with metastatic colon cancer. Several other studies reported the prognostic significance of markers of necroptosis in pancreatic and cervical cancer [23,24].

Although the statistical power of our investigation is limited by the small cohort size, the current retrospective study suggests that patients with high expression of RIPK3

in the primary tumor could benefit mostly from treatment with 5-FU based first-line chemotherapy. A significant limitation of our study is the absence of information on whether the expression level of RIPK3 in pretreatment biopsy samples correlates with the expression of all metastatic sites.

Despite these limitations, this study opens a gateway for the investigation of RIPK3 as potential marker to identify appropriate patients for a new pro-necroptotic therapy in clinical trials to improve patient outcomes.

References

- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin*. 2011;61(2):69-90.
- Van Cutsem E, Cervantes A, Nordlinger B, Arnold D, Group EGW. Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2014;25 Suppl 3:iii1-9.
- Zhao H, Jaffer T, Eguchi S, Wang Z, Linkermann A, Ma D. Role of necroptosis in the pathogenesis of solid organ injury. *Cell Death Dis*. 2015;6:e1975.
- Linkermann A, Green DR. Necroptosis. *N Engl J Med*. 2014;370(5):455-65.
- He S, Wang L, Miao L, Wang T, Du F, Zhao L, et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell*. 2009;137(6):1100-11.
- Fulda S. The mechanism of necroptosis in normal and cancer cells. *Cancer Biol Ther*. 2013;14(11):999-1004.
- Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell*. 2009;137(6):1112-23.
- Oliver Metzger M, Fuchs D, Tagscherer KE, Grone HJ, Schirmacher P, Roth W. Inhibition of caspases primes colon cancer cells for 5-fluorouracil-induced TNF-alpha-dependent necroptosis driven by RIP1 kinase and NF-kappaB. *Oncogene*. 2016;35(26):3399-409.
- Voigt S, Philipp S, Davarnia P, Winoto-Morbach S, Roder C, Arenz C, et al. TRAIL-induced programmed necrosis as a novel approach to eliminate tumor cells. *BMC Cancer*. 2014;14:74.
- Ishibashi H, Suzuki T, Suzuki S, Moriya T, Kaneko C, Takizawa T, et al. Sex steroid hormone receptors in human thymoma. *J Clin Endocrinol Metab*. 2003;88(5):2309-17.
- Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst*. 2000;92(3):205-16.
- Fakih MG. Metastatic colorectal cancer: current state and future directions. *J Clin Oncol*. 2015;33(16):1809-24.
- Cho YS. Perspectives on the therapeutic modulation of an alternative cell death, programmed necrosis (review). *Int J Mol Med*. 2014;33(6):1401-6.
- Winder T, Lenz HJ. Molecular predictive and prognostic markers in colon cancer. *Cancer Treat Rev*. 2010;36(7):550-6.
- Popat S, Matakidou A, Houlston RS. Thymidylate synthase expression and prognosis in colorectal cancer: a systematic review and meta-analysis. *J Clin Oncol*. 2004;22(3):529-36.
- Wang W, Cassidy J, O'Brien V, Ryan KM, Collie-Duguid E. Mechanistic and predictive profiling of 5-Fluorouracil resistance in human cancer cells. *Cancer Res*. 2004;64(22):8167-76.
- Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ*. 2009;16(1):3-11.
- Ricci MS, Zong WX. Chemotherapeutic approaches for targeting cell death pathways. *Oncologist*. 2006;11(4):342-57.
- Kanduc D, Mittelman A, Serpico R, Sinigaglia E, Sinha AA, Natale C, et al. Cell death: apoptosis versus necrosis (review). *Int J Oncol*. 2002;21(1):165-70.
- Su Z, Yang Z, Xie L, DeWitt JP, Chen Y. Cancer therapy in the necroptosis era. *Cell Death Differ*. 2016;23(5):748-56.
- Koo GB, Morgan MJ, Lee DG, Kim WJ, Yoon JH, Koo JS, et al. Methylation-dependent loss of RIP3 expression in cancer represses programmed necrosis in response to chemotherapeutics. *Cell Res*. 2015;25(6):707-25.
- Moriwaki K, Bertin J, Gough PJ, Orlowski GM, Chan FK. Differential roles of RIPK1 and RIPK3 in TNF-induced necroptosis and chemotherapeutic agent-induced cell death. *Cell Death Dis*. 2015;6:e1636.
- Ruan J, Mei L, Zhu Q, Shi G, Wang H. Mixed lineage kinase domain-like protein is a prognostic biomarker for cervical squamous cell cancer. *Int J Clin Exp Pathol*. 2015;8(11):15035-8.
- Colbert LE, Fisher SB, Hardy CW, Hall WA, Saka B, Shelton JW, et al. Pronecrotic mixed lineage kinase domain-like protein expression is a prognostic biomarker in patients with early-stage resected pancreatic adenocarcinoma. *Cancer*. 2013;119(17):3148-55.