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ORIGINAL ARTICLE

The Trend of *ripk1*/*ripk3* and *mlkl* Mediated Necroptosis Pathway in Patients with Different Stages of Prostate Cancer as Promising Progression Biomarkers

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SUMMARY

Background: **Programmed cell death is critical to maintain tissue homeostasis. Necroptosis, as well as apoptosis, has been considered as another form of regulated cell death which can be used as an effective way to overcome apoptosis-resistant tumor tissue growth. The aim of present study was to test whether or not *ripk1*, *ripk3*, or *mlkl* expression levels, as the key necroptotic modulators in different stages of prostate tumor growth.**
Methods: **Sixty-seven prostate tissues representing histologically confirmed cancer were selected. The cancer samples were categorized into 4 different stages based on cellular differentiation, tumor growth rate, and extra tissue expansion to regional lymph nodes, average PSA levels, and tumor volume. RNA extraction, cDNA synthesis and quantitative real time PCR were done based on standard guidelines.**
Results: **No statistically significant changes in *ripk1* expression showed in all three stages (stage II to IV). The expression pattern of *ripk3* represented a remarkable elevation in early stage, while, predominantly repressed in final cancer stage (IV). Also, there has been a significant negative correlation between *ripk3* gene expression and tumor size and PSA levels.**
Conclusions: **We cannot exclude the importance of the key regulator proteins in development and progression of prevalent lethal disease like prostate cancer. The *ripk1*/*ripk3* mediated necroptosis pathway is more activated in early stages of prostate cancer via induced *ripk3* expression, while repressed during prostate cancer final stages. Also, the repression of *ripk3* is related to elevation of both PSA levels and tumor volume which represented the tumor progression in final stages.**
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INTRODUCTION

Cell death is one of the key physiological processes which is critical for maintaining tissue homeostasis including, growth, volume, and even tissue degeneration [1]. This phenomenon is associated with highly con-

served molecular and morphological changes in which one or several events have occurred including loss of plasma and mitochondrial membrane integrity, cell fragmentation, engulfment by adjacent cells, and phosphatidylserine flip flop to the outer membrane. Cell death processes have been divided into the four distinct following types including, apoptosis, necrosis, autophagy, and cornification [2]. While traditionally, necrosis was defined as an accidental or unregulated form of cell death, apoptosis and autophagy were classified as cell suicide and regarded as programmed cell death [3,4]. However, this view has been altered recently introducing several forms of regulated necrosis, including phosphoribosyl pyrophosphate (PPRP)-1-mediated necrotic death, pyroptosis, ferroptosis, p53-mitochondrial mediated necrosis, and necroptosis [5-9].

Necroptosis has recently gained increasing attention due to the fact that there have been several overlapped upstream signaling elements with apoptosis. Necroptosis occurs when the cells suffer from severe stress or are treated with chemotheric agents or inflammatory factors that represented necrotic cell death morphology and activation of autophagy [10,11]. Moreover, in cancer stem cells, several mechanisms have occurred to circumvent growth suppression by apoptosis [12].

Prostate cancer is the second most abundant cancer among males and one of the leading causes of death worldwide. Surgery, radiation therapy, and finally androgen-deprivation therapy are taken depending on the stages to overcome cellular growth. Nevertheless, most tumors will recur in about two years, converting to castration-resistant prostate cancer (CRPC) which would be incurable. Although CRPC cell populations are resistant to androgen-deprivation, they are still susceptible to programmed cell death stimuli like apoptosis [13]. TNF-related apoptosis-inducing ligand (TRAIL) and tumor necrosis factor-alpha (TNF-α) have been suggested to play critical roles in prostate cancer hemostasis [1, 13]. TNF is believed to be secreted from malignant epithelial cells and, in spite of tumor cell death stimulation, TNF acts as an autocrine survival signal for cancer cells, resulting in promoting metastasis, invasion and regulating infiltration of macrophages into the tumor microenvironment [14]. In contrast, tumor necrosis factor (TNF) is believed to initiate two cell death pathways, caspase-8-dependent apoptosis and *ripk1*/3 kinase-dependent necroptosis [15]. It has been reported that *ripk1*, *ripk3*, or *mlkl* knockouts, the three key regulators of necroptosis agents in cancer cells, reduce their proliferation *in vitro* and tumor formation ability *in vivo* [16]. Herein, the expression level of these three genes are investigated in patients with diagnosed prostate cancer in three different levels to determine the susceptibility of tumors to necroptosis in progression of prostate cancer as a therapeutic target for chemotherapy strategies.

MATERIALS AND METHODS

Sample collection

A total of 67 patients with prostate cancer history and 16 normal tissues as control ranging from 55 to 70 years old, who were admitted to Hasheminejad Kidney Center were selected. Informed consent was obtained from all patients, and the study was approved by the ethical committee of Iran University Research Ethics Committee.

Patients were classified with regard to three factors including TNM (tumor, node, metastasis) staging system, serum concentration of PSA, and tumor volume stages according to the protocol established by the European Association of Urology. According to this protocol, prostate tumors are classified into 4 distinct stages including, (A) stage I: Early stage level of cancer growth with no tumor expansion (which cannot be felt one-half of 1 side of the prostate or even less than that). Due to the early stage, the cancer cells are well differentiated, without metastasis to regional lymph nodes and average PSA levels are less than 10 ng/mL. (B) stage II: The tumor is found only in the prostate although it may have an increasing risk of fast growth and spreading. PSA levels are moderate or low. Metastasis to the regional lymph nodes has not occurred, and average PSA levels reach 10 and 20 ng/mL. (C) stage III: the tumor is quickly growing with PSA levels greater than 20 ng/mL, but metastasis has not occurred and the tumor has not spread to regional lymph nodes. (D) stage IV: The cancer has spread out from prostate to regional lymph nodes and PSA levels are high with any rates.

Quantitative PCR procedure

Primarily, prostate tissue samples were collected via radical prostatectomy surgery and immediately stored in cryotubes containing RNA later solution (Qiagen, Hilden, Germany) at -20°C. After collecting all samples, RNA later solution samples were removed, tissues were homogenized, and total RNA was extracted using TRIZOL reagent via previously described methods [17]. The cDNA synthesis was performed via smART First Strand cDNA Synthesis Kit (EURx Company, Poland). Then, the expression levels of three genes, *ripk1*, *ripk3*, and *mlkl* genes in prostate tissues were detected using quantitative PCR using specific primers (Table 1). Finally, real-time PCR was done using SYBR green dye as the detection signal.

Statistical analysis

Statistical analyses were done using SPSS 18 (Chicago, IL, USA). A “p-value” equal or less than 0.05 was accepted as statistically significant.

Table 1. Specific primers for quantitative determination of *ripk1*, *ripk3* and *mlkl* genes in prostate tissues.

Genes	Primer sequence	Tm
mlkl	5′- GGC AAG GAG ACA GAA CTC AA-3′	59
	5′- CAC AGA GAT CCA GTT GCA GA-3′	
ripk1	5′- AGTCGAGACTGAAGGACACAGCACT-3′	58
	5′- TCCAGCAGGTCACTGGATGCCAT-3′	
ripk3	5′- CTTGAACCCTCCGCTCCTGC-3′	58
	5′- AATCTGCTAGCTT GGCGTGG-3′	
β-actin	5′- TGG GCA TCC ACG AAA CTA C-3′	57
	5′- GAT CTC CTT CTG CAT CCT GT-3′	

Table 2. Expression patterns of *ripk1*, *ripk3* and *mlkl* genes in different stages of prostate tissues.

	Fold change					
	ripk1	p-value	*ripk3*	p-value	*mlkl*	p-value
Control	1.00 ± 0.00	0.014	1.00 ± 0.00	< 0.001	1.00 ± 0.00	0.101
Stage II	0.69 ± 1.48		2.76 ± 3.72		1.03±1.57	
Stage III	1.41 ± 3.00		0.69 ± 1.76		0.91±1.08	
Stage IV	1.74 ± 1.59		0.14 ± 0.08		1.71±1.44	

Table 3. Correlation between expression levels of *ripk1* and *ripk3* in different stages compared to control.

Dependent variable	(I) stage	(J) stage	Mean difference (I - J)	Std. error	Sig.
ripk3	control	Stage II	-1.7656789 *	0.6208348	0.043
		Stage III	0.3045899	0.3585735	0.946
		Stage IV	0.8632857 *	0.0310405	0.000
ripk1	control	Stage II	0.3103125	0.2612720	0.796
		Stage III	-0.4148000	0.6133042	0.982
		Stage IV	-0.7457143	0.6032091	0.762

The mean difference is significant at the 0.05 level.

RESULTS

To investigate the expression levels of the *ripk1*, *ripk3*, and *mlkl* genes in patients with prostate cancer, 16 normal and 67 cancer tissue samples were analyzed. The cancerous samples were categorized into three stages based on the growth rate, local extent of the prostate tumor, whether the cancer had spread to nearby regional nodes or metastasized to distant parts of the body, serum PSA levels, and tumor volume. All patients were between the ages of 50 and 70. Kruskal-Wallis H, one-way ANOVA analysis was used to examine homogeneity of age variables among the groups. The age difference between groups (stage 2, stage 3, stage 4, control) was not meaningful p-values of 0.273 (p-value > 0.05), hence, all the groups were homogeneous in age.

In the following analysis, different cancer stages were tested including 16 normal tissue samples, 34 patients with stage II (52.31%), 24 patients with stage III (36.92%), and 7 patients with stage III (10.78%). As mentioned in Table 2, the expression level of the *ripk1*, *ripk3*, or *mlkl* genes among prostate tissues containing normal and cancerous samples within all stages groups is presented. Kruskal-Wallis test was used to compare

Table 4. Correlation between tumor volume and PSA levels in prostate tumor progression.

Correlation			Tumor volume	PSA levels
Spearman's rho	*ripk1*	Correlation coefficient	-0.094	-0.093
		Sig. (2-tailed)	0.450	0.457
		n	67	67
	ripk3	Correlation coefficient	-0.275 *	-0.262 *
		Sig. (2-tailed)	0.024	0.032
		n	67	67
	mlkl	Correlation coefficient	-0.024	-0.017
		Sig. (2-tailed)	0.848	0.890
		n	67	67
	Tumor volume	Correlation coefficient	1.000	0.559 *
		Sig. (2-tailed)	0.00	0.000
		n	67	67

*** - Correlation is significant at the 0.05 level (2-tailed).**

variables (*ripk1*, *ripk3* and *mlkl*) in different tested groups (stage 2, stage 3, stage 4, control). According to the results, both *ripk1* and *ripk3* showed a significant change within stages with p-values of 0.014 and < 0.001, respectively. However, for *mlkl* gene, (p-value = 0.101), Kruskal-Wallis test showed no significant changes during mentioned stages.

Moreover, to take a brief look at these three targeted genes, *ripkl, ripk3* and *mlkl* genes showed different patterns within stages compared to control. As illustrated in Table 3, according to Dannet's T3 Test between each cancerous stages and control, *ripk1* showed significant lower levels in stage II, while during cancer progression, there was a positive trend on the gene expression that is only significant in stage IV. By contrast, *ripk3* showed dramatic expression levels in stage II, while in the following stages (stage III and IV), it showed a decrease in gene expressions, which showed no statistically significant changes compared to control.

Also, the correlation between *ripk1*, *ripk3*, and *mlkl* gene expression as well as prostate tumor volume and PSA levels was tested. According to the obtained data (see Table 4), there is a strong positive correlation between tumor volume and PSA levels which shows the elevation of PSA levels in prostate tumor progression. Moreover, there is no significant correlation between *ripk1* and *mlkl* expression with cancer progression, but there is a significant negative correlation between both tumor volume and PSA concentration with *ripk3* expression levels. In this propose, the expression levels were reduced during cancer progression.

DISCUSSION

Programmed cell death has provided a great way into finding new ways for cancer diagnosis and therapy [12, 18,19]. There have been several types of signaling platforms in cells that can initiate cell death including; the death-inducing signaling complex (DISC), TNF complex II, apoptosome, PIDDosome, and ripoptosome [15, 20]. Necroptosis, the newly identified caspase-independent programmed cell death, was reported to play a critical role in tumorigenesis as a backup cell death mechanism in cancer cells [21]. Necroptosis can be triggered by a range of stimuli, like viral infection, cytokines, chemicals, damage-associated molecular patterns (DAMPs), and several forms of physicochemical cellular stresses like reactive oxygen species (ROS) [22]. The dominant pathway leading to necroptosis is initiated by TNF-TNFR1 complex formation which results in cell survival, apoptosis or necroptosis [23]. Most studies mentioned that necroptosis is triggered via the *rip1-rip3-mlkl* complex called "necroptosome" [15], and simultaneously, apoptosis would be blocked via NF-κB mediated overexpression of cellular FLICE inhibitory protein (cFLIP) [24,25].

Our investigations demonstrate for the first time that during prostate cancer development, the dominant active pathway is mainly shifted from triggered necroptosis pathway in stage II to inhibitory platform in final stage IV. Briefly, in early stages of prostate cancer, *ripk3* expression elevated remarkably without significant changes in *mlkl* and *ripk1* RNA contents. By contrast, the progression of cancer to stage III and IV lead to repression of *ripk3* gene expression. It has been well known that *ripk3* has a key role in the necroptosis induced pathway which predominantly induces inflam-

mation as a cancer-fueling agent resulting in cancer extravasation and metastasis in cancer, besides escaping from the apoptosis pathway [26]. Similar to previous investigations, our results showed that in early stages of prostate cancer, necroptosis is the main pathway to induce inflammation microenvironment and metastasis [26]. Reduced expression of *ripk3* has been reported in several cancers in different stages. For instance, in primary colorectal cancers, CD34+ acute myeloid leukemia, and breast carcinoma regardless of tumor subtype [27-30]. In contrast with our results, some others mentioned the role of *rip1*/*ripk1* overexpression as an oncogenic regulator in the progression and survival of cancers like melanoma, human non-small cell lung cancer, pancreatic ductal adenocarcinoma, and glioblastoma cells. It has been revealed that the proliferative effects of *ripk1* overexpression were mediated by NF-κB activation [31-35]. Also, kinase activity (not expression levels) of *ripk1* but not *ripk3* is critical for extravasation step of metastasis [36]. Moreover, our investigation represented that there is a negative correlation between *ripk3* expression and prostate cancer progression that is measured by two factors including PSA levels and tumor volume. The opposite trend is reported in esophageal xenograft tumor bearing mice with *ripk3* overexpression that showed cellular growth suppression [37].

CONCLUSION

According to the current investigation, in early stages of prostate cancer, the *ripk3* overexpression promotes the cells for extravasation and apoptosis pathway resistance, while in late stages, tumor cells growth was dominant due to the *ripk3* repression. From an overall point of view, current studies surrounding this controversy on introducing a trend of dominant pathways in different prostate cancer stages as the prognostic biomarker for suitable therapeutic propose.

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Ethical Approval:
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Declaration of Interest:
The authors declare that they have no competing interest.

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