THE EFFECT OF LENTINAN ON THE GENE EXPRESSION OF MAJOR ION CHANNELS IN T LYMPHOCYTES ISOLATED FROM MICE EXPOSED TO CHRONIC RADIATION

by

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It is known that Kv1.3, KCa3.1, CRAC, TRPM7, and Clswell are five important ion channels in T lymphocytes. To investigate the mechanism underlying the protective effects of lentinan against T lymphocytes injury induced by chronic radiation in mice, we determined the expression profiles of Kv1.3, KCa3.1, Orai1, STIM1, TRPM7, and Clswell genes. Mice were injected with different concentrations of lentinan before exposure to ^{60}Co γ-ray. Total RNA was then extracted from T lymphocytes in murine spleen. The Real time-PCR was performed to detect the expression levels of Kv1.3, KCa3.1, Orai1, STIM1, TRPM7, and Clswell genes. The results show that, out of the six ion channels gene expression detected in the present study, only Kv1.3 and Orai1 were altered by lentinan in mice under chronic radiation stress. Kv1.3 and Orai1 expression were significantly elevated in T-lymphocytes isolated from irradiated mice, and were normalized by lentinan treatment. Our results suggest that that Kv1.3 and Orai1 channels may mediate the anti-radiation effects of lentinan.

Key words: lentinan, T lymphocyte, Kv1.3, orai1, chronic radiation stress

INTRODUCTION

It is well recognized that ionizing radiation, even at low levels, is harmful to people. Cho *et al*. [1] have found that low doses of ionizing radiation significantly induced higher centromere negative micronuclei in radiographers when compared to healthy subjects. Not only that ionizing radiation is harmful to radiographers, but also to the cancer patients receiving radiation therapy. During the treatment process of cancer by radiation, both cancer and normal cells will be killed. A close association between radiation and T lymphocyte injury has been demonstrated. The radiation was shown to induce apoptosis in T lymphocytes dose-independently [2]. In addition, Mark *et al*. have proposed that CD^{4+} T lymphocytes may bridge immunostimulatory and radiosensitizing strategies [3].

The Kv1.3, KCa3.1, CRAC (Orai1+STIM1), TRPM7, and Cl_{swell} are the five important ion channels in T lymphocytes, necessary to perform functions vital for sustaining cell homeostasis and T lymphocytes activation [4]. Numerous studies have shown that the aforementioned ion channels play pivotal roles in differentiation and plasticity of T lymphocytes, which is important during the activation of the immune system.

The Kv1.3, a voltage-gated potassium channel has been proven to play an important role in Ag-specific CD^{4+} T cells in autoimmune encephalomyelitis [5]. Bobak *et al*. also found that Kv1.3 plays a central role in the osmoregulation of T lymphocytes under different conditions [6]. The KCa3.1, a Ca^{2+}-activated K^+ channel expressed in various tissues, participates in the regulation of cell proliferation [7] and gastric acid secretion [8]. The Orai1 and STIM1 (stromal interacting molecule 1) are Ca^{2+}-release activating Ca^{2+} channels (CRAC) which modulate store-operated Ca^{2+} entry. The TRPM7, a transient receptor potential channel, contributes to lymphocyte function potentially through regulating magnesium homeostasis in various immune responses [9]. The Cl_{swell} was shown to play a unique role in regulation of polymorphonuclear leukocyte chemotaxis [10]. In summary, these ion channels are important in modulating T lymphocyte functions. Therefore we hypothesized that the anti-radiation effects of lentinan may be associated with normalization of ion channels in T lymphocytes.

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The lentinan, isolated from Shiitake, is a polysaccharide drug with a complex conformation [11]. It was demonstrated to possess anti-cancer, anti-viral, immunomodulatory and other properties [12,13]. It is now applied to treat several kinds of cancer as a supplementary drug [14-16]. However, the underlying mechanism of the protective effect of lentinan against chronic radiation injury is poorly understood. Recently, we reported that lentinan is protective against T lymphocyte injury in mice under chronic radiation stress [17]. In the present study, we determined the gene expression of the major ion channels in T lymphocytes from murine spleen to delineate the role of those ion channels in anti-radiation effects of lentinan.

MATERIALS AND METHODS

Animals

The Male Kunming mice (6~8 weeks; 18~22 g) were purchased from Experimental Animal and Animal Studies Center of Qingdao, China. Upon arrival, mice were kept separately in standard plastic cages under the following conditions: temperature of 20-22 °C and humidity of 40-60%. Mice were given free access to Purina chow and water. All experiment procedures performed on the mice were in accordance with the regulations laid down by Chinese Association for Laboratory Animal Science. The mice license number was SCXK(Lu)20090007.

Drugs

The lentinan (1 mg/2 ml) was purchased from Jinling Pharmaceutical Co., Ltd., China (Country medicine accurate character H44021772). The Lentinan was further diluted to 0.002 mg/ml, 0.01 mg/ml, and 0.02 mg/ml with saline buffer solution before the injection.

Chemicals

The RPMI 1640 and fetal calf serum (FCS) were purchased from Tianjin Haoyang Biological Manufacture Co., Ltd., China and Zhejiang Tianhang Biological Technology Co., Ltd., China, respectively. The StarSpin animal mRNA kit was purchased from Genstar Biosolutions Co., Ltd., China. The PrimeScript$^{(R)}$ RT reagent kit with gDNA Eraser and the SYBR$^{(R)}$ Premix Ex TaqTM was purchased from Takara Biotechnology (Dalian) Co., Ltd., China.

Experimental design

The mice were randomized into five groups (n = 10 each), which consisted of mice exposed to radiation (Rad), normal control mice (Ctr), irradiated mice treated with lentinan in low (0.002 mg/ml, Rad+LLTN), middle (0.01 mg/ml, Rad+MLTN), and high (0.02 mg/ml, Rad + HLTN) concentrations. Mice from all groups, except those in the control group, were exposed to systemic radiation of 0.4 Gy ^{60}Co γ-ray continuously from Monday to Friday for six weeks. The mice from the three lentinan treament groups were injected intraperitoneally with 0.5 ml of lentinan at concentrations mentioned above before each radiation.

Cell culture

After the last radiation, the mice were euthanized by cervical vertebra dislocation, and soaked in 75% ethanol for 30 seconds. The spleens were removed and then smashed in the culture medium with 10 ml syringe plunger, followed by centrifugation at 265 g/min for 15 minutes. After removal of the supernatant, red blood cell lyses buffer was added to the centrifuge tube, followed by centrifugation (265 g/min, 15 min). After discarding the supernatant, T lymphocytes were recovered from the pellet, which contained different populations of lymphocytes, using nylon wool fiber column T, as described previously [16].

Total RNA preparation and primer design

After culturing the T lymphocytes at 37 °C, 5% CO_2 for 14 h, a total RNA was extracted with a starspin animal mRNA kit according to the manufacturer's instruction. Concentration and purity of the total RNA samples were determined by reading the absorbance at 260 nm and 280 nm.

The primers of the six ion channel protein genes were designed by UCSC Genome Browser. The molecular characteristics of Cl_{swell} remain unclear [4]. Since Cl_{swell} may be a homolog of TMEM16A [18], we designed the primers for Cl_{swell} based on the sequence of TMEM16A mRNA . The sequences of the primers are shown in tab. 1.

RT-q PCR

The cDNA were synthesized using PrimeScript$^{(R)}$ RT reagent Kit according to the manufacturer's instruction. The PCR reaction mixture contained 12.5 l SYBR$^{(R)}$ Premix Ex Taq^{TM}, 5 M of PCR Forward Primer and PCR Reverse Primer, 2 l cDNA and 9 l dH_2O, adding up to a final volume of 25 l. The PCR conditions of the six ion channel genes and GAPDH are listed in tab 2. The GAPDH was selected as a house-

Table 1. Primer sequences for the expression study of the six major ion channel genes in T lymphocytes

Six ion channels genes	Prime sequence	Accession no.
Kv1.3	F: CTGGTGGGCAGTAGTAACCATGA R: ACACCTGCGATGGCACAAAG	NM_008418.2
KCa3.1	F: ATTCCGATCACATTCCTGACCA R: GCCTTGTTGAACTCCAGCTTCC	NM_008433.4
ORAI1	F: CGCCAAGCTCAAAGCTTCC R: CAGGCACTAAAGACGATGAGCA	NM_175423.3
STIM1	F: TGAGGCCGTCCGAAACATC R: TCACTGTTGGGTCATGGTAATTGAG	NM_009287.4
TRPM7	F: CTGCCAATCTAGGAGAAGATGCAAT R: AGGCGTGTAGTCATTCCTCTTCAAA	NM_001164325.1
Cl_{swell}	F: CAGTTGTGCCTAGCGCTCATATTTA R: TCCCTGCAATGGCTCCAA	Q8BHY3.2
GAPDH	F: TGTGTCCGTCGTGGATCTGA R: TTGCTGTTGAAGTCGCAGGAG	NM_008084.2

Table 2. Cycling conditions of PCR for each gene

Six ion channels genes	Product size	Cycling conditins	Number of cycles
Kv1.3	MA095989-F 23 bp MA095989-R 20 bp	1.95 °C 30 s 2.95 °C 5 s 55 °C 30 s 72 °C 60 s	Process 2 with 35 cycles
KCa3.1	MA106106-F 22 bp MA106106-R 22 bp	1.95 °C 30 s 2.95 °C 5 s 55 °C 30 s 72 °C 30 s	Process 2 with 35 cycles
ORAI1	MA096721-F 19 bp MA096721-R 22 bp	1.95 °C 30 s 2.95 °C 5 s 53 °C 30 s 72 °C 60 s	Process 2 with 35 cycles
STIM1	MA069841-F 19 bp MA069841-R 25 bp	1.95 °C 30 s 2.95 °C 5 s 60 °C 30 s 72 °C 60 s	Process 2 with 40 cycles
TRPM7	MA126510-F 25 bp MA126510-R 25 bp	1.95 °C 30 s 2.95 °C 5 s 55 °C 30 s 72°C 30 s	Process 2 with 35 cycles
Cl_{swell}	MA120495-F 25 bp MA120495-R 25 bp	1.95 °C 30 s 2.95 °C 5 s 53 °C 30 s 72 °C 30 s	Process 2 with 35 cycles
GAPDH	MA050371-F 20 bp MA050371-R 21 bp	1.95 °C 30 s 2.95 °C 5 s 60 °C 60 s	Process 2 with 30 cycles

keeping gene because its expression level was stable in normal lymphoblastoid cells [19]. The specificity of PCR amplification was analysed by melting curve.

Each sample was run in duplicate in TL988 Real Time Quantitative PCR (Xi'an Tianlong Science and Technology Co., Ltd). The threshold cycle, Ct, was calculated as Ct (target gene) – Ct (GAPDH). The relative changes in target gene in different treatment groups were determined by the formula $2^{-\ Ct}$, where Ct = Ct (control) – Ct (treatment group).

Statistical analysis

The experimental data were expressed as mean ± SD and analyzed using ANOVA (IBM SPSS software, 19.0v). The LSD-*t test* was applied to analyze the difference between the individual groups. The $p < 0.05$ was considered as statistically significant.

RESULTS

Effects of lentinan on Kv1.3 expression in T lymphocytes isolated from irradiated mice

As shown in fig 1, the mRNA expression of Kv1.3 in T lymphocytes isolated from Rad group was up-regulated by 3.19 folds when compared to Ctr group ($p < 0.05$). Interestingly, treatment with lentinan at lower (Rad+LLTN) and higher (Rad+HLTN) doses,

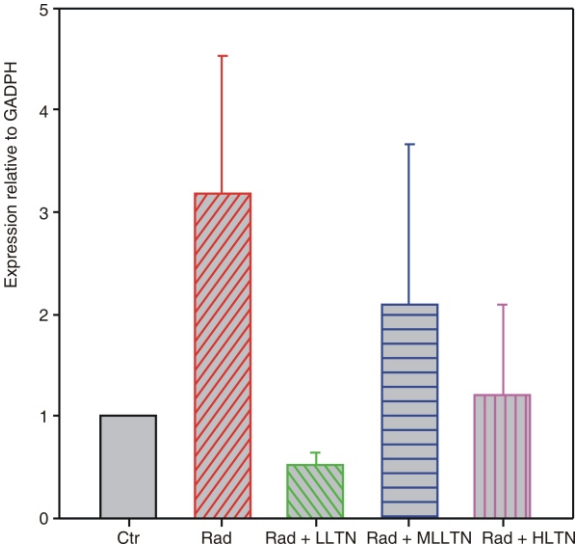

Figure 1. Relative quantification of mRNA expression of Kv1.3 in T lymphocytes isolated from different groups
In Rad group, Kv1.3 expression was increased by 3.18 times when compared to Ctr group (p = 0.003). The treatment with low and high doses of lentinan effectively attenuated the effect of radiation on this ion channel

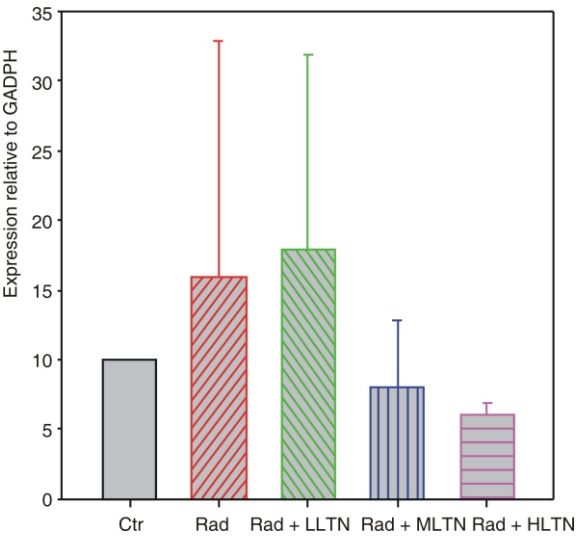

Figure 2. Relative quantification of mRNA expression of KCa3.1 in T lymphocytes isolated from different groups
The gene expression of KCa3.1 did not differ between Ctr, Rad, Rad + LLTN, Rad + MLTN and Rad + HLTN groups

but not the middle dose (Rad+MLTN), significantly ameliorated the increased Kv1.3 levels induced by radiation. The data suggest that Kv1.3 may mediate the protective effect exerted by lentinan on T lymphocyte injury induced by chronic radiation.

Effects of lentinan on KCa3.1 expression in T lymphocytes isolated from irradiated mice

We further investigated the effects of lentinan on KCa3.1 expression in T lymphocytes isolated from irradiated mice. As shown in fig. 2, radiation stress, with or without lentinan treatments, did not significantly alter the mRNA expression of KCa3.1.

Effects of lentinan on Orai1 expression in T lymphocytes isolated from irradiated mice

To investigate whether Orai1 channel plays a role in T lymphocyte injury, we measured the mRNA expression of the ion channel in T lymphocytes injured by radiation. Interestingly, Orai1 was up-regulated in response to chronic radiation stress in comparison with Ctr group ($p < 0.05$) (fig. 3). Low and medium concentrations of lentinan did not normalize the expression of Orai1 in injured T lymphocytes. However, treatment with a high dose of lentinan significantly abolished the effect of radiation on Orai1 expression ($p < 0.05$).

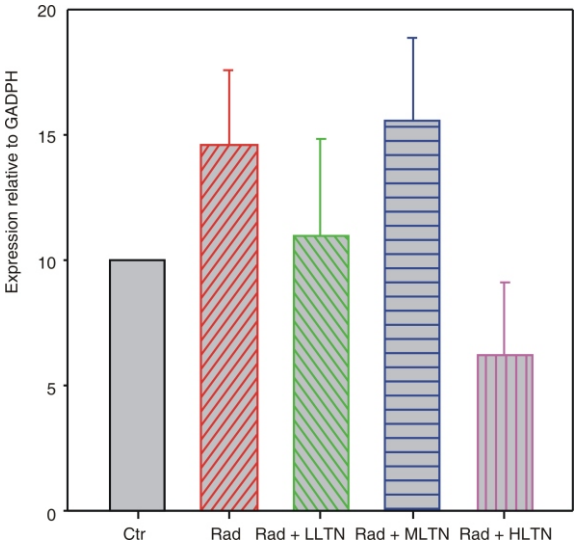

Figure 3. Relative quantification of mRNA expression of Orai1 in T lymphocytes isolated from different groups
The Orai1 expression in T lymphocytes extracted from Rad group was significantly higher than that of Ctr group (p = 0.043), which was normalized in Rad + HLTN group (p = 0.001)

Effects of lentinan on STIM1 expression in T lymphocytes isolated from irradiated mice

To determine whether lentinan protects T lymphocytes from radiation by regulating STIM1 expression, we also measured the gene expression of the latter in T lymphocytes isolated from all experimental

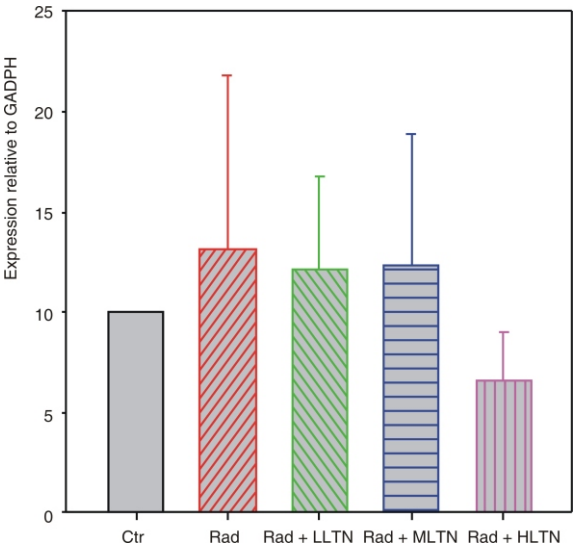

Figure 4. Relative quantification of mRNA expression of STIM1 in T lymphocytes isolated from different groups
The gene expression of STIM1 did not differ between Ctr, Rad, Rad + LLTN, Rad + MLTN and Rad + HLTN groups

groups. As depicted in fig. 4, there was no significant difference among all the groups, suggesting that STIM1 does not mediate the protective effect of lentinan against chronic radiation.

Effects of lentinan on TRPM7 expression in T lymphocytes isolated from irradiated mice

The mRNA expression of TRPM7, another important ion channel associated with T lymphocyte activation, was also examined. The results showed that TRPM7 mRNA expression in T lymphocytes isolated from irradiated mice was increased by 2.3 folds when compared to control group. Treatment with lower and higher doses of lentinan have a tendency to attenuate the effect of radiation on TRPM7 expression (1.27 and 1.37 folds *vs*. control group, respectively). However the differences were not statistically significant due to the variations within the group (fig. 5).

Effects of lentinan on Cl_{swell} expression in T lymphocytes isolated from irradiated mice

To understand the effects of lentinan on Cl_{swell} expression in T lymphocytes injured by chronic radiation, we examined the gene expression level of Cl_{swell} with TMEM16A primers. Figure 6 shows that Cl_{swell} expression was not altered in Rad and Rad + LLTN groups ($p > 0.05$), but was significantly elevated for medium and high doses of lentinan.

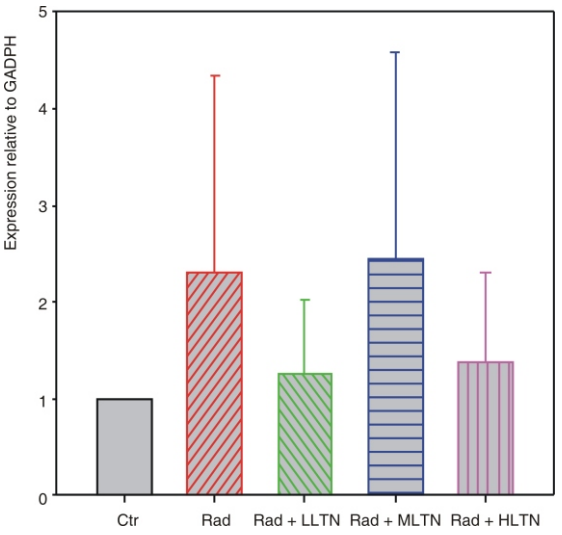

Figure 5. Relative quantification of mRNA expression of TRPM7 in T lymphocytes isolated from different groups
The gene expression of STIM1 did not differ, statistically, between Ctr, Rad, Rad + LLTN, Rad + MLTN and Rad + + HLTN groups

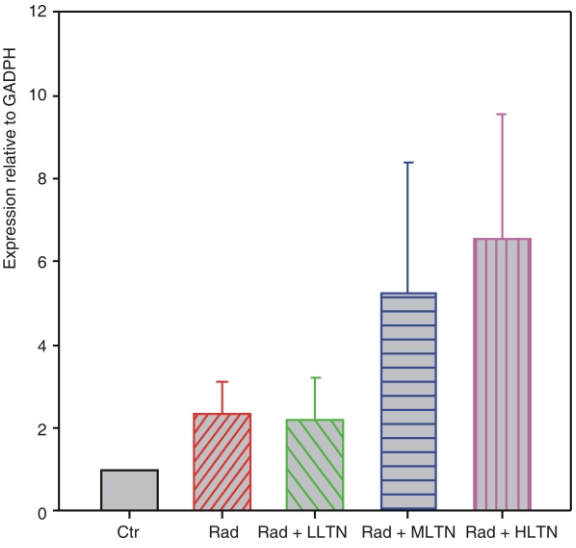

Figure 6. Relative quantification of mRNA expression of TRPM7 in T lymphocytes isolated from different groups
The Cl_{swell} expression was not affected by radiation, but was significantly elevated by the medium and high doses of lentinan treatment

DISCUSSION

In a recent review, Borchiellini *et al*. [20] have demonstrated DNA damage in response to radiation therapy. Moreover, cancer patients undergoing radiation therapy may face the risk of late complication, which is associated with CD^{4+} and CD^{8+} lymphocytes apoptosis [21], injuring T lymphocytes to some extent. Ever since the discovery of lentinan, many studies have reported that it may exert pharmacological effects in different pathological conditions [14, 22].

Roupas *et al*. have summarized the role of active compounds extracted from edible mushrooms, including lentinan, in health outcomes [23]. According to our results [17], as well as other studies [24, 25], we speculate that the therapeutic effects of lentinan are comprised of an intricate network. Anti-cancer and anti-radiation effects of lentinan may be related to its immunoregulation effect.

In this study, we investigated the mechanism of protective effects of lentinan on T lymphocytes isolated from mice under chronic radiation by examining the gene expression profiles of Kv1.3, KCa3.1, Orai1, STIM1, TRPM7, and Cl_{swell} in T lymphocytes from control and irradiated mice, with or without different doses of lentinan treatments. The Kv1.3 potassium channel played a key role in T cell function. It participated in the maintenance of calcium-influx during the process of lymphocytes activation, presenting a possible target for selective immunomodulation [26]. Our results showed that Kv1.3 can be influenced by lentinan treatments, suggesting that Kv1.3 channel may be a pharmacological target of lentinan. The Ca^{2+} activated K^+ channel K(Ca)3.1, expressed in human lung mast cells and human fibrocytes, was proposed to modulate the cell migration [27, 28]. The KCa3.1 expressions in T lymphocytes isolated from Rad, Ctr, Rad + LLTN, Rad + MLTN, and Rad + HLTN groups did not differ from each other, suggesting that KCa3.1 plays no role in the T lymphocytes injured by chronic radiation.

The Orai1 and STIM1, both are classified as CRAC (Ca^{2+} release-activated Ca^{2+} channels), opened upon release of Ca^{2+} from intracellular stores. These two proteins are particularly important in T lymphocytes activation, which link between the signal transduction cascade and changes of genes expression [29]. It has been suggested that, in the case of T-cell activation, Ca^{2+} modulates the progression of the cell-cycle by inducing gene transcription [30]. The Orai1, a plasma membrane-resident Ca^{2+} channel, was shown to mediate Ca^{2+} entry [31]. The STIM1 is a recently discovered ion channel which plays an essential and unique role in store-operated Ca^{2+} entry. Both of these ion channels are related to a range of cell functions, including proliferation, adhesion and gene expression [32]. In the present study, T lymphocytes Orai1 gene expression, which was up-regulated by chronic radiation, was ameliorated by the intervention of lentinan. The TRPM7, a Ca^{2+} and Mg^{2+} permeable ion channel was shown to regulate polarized cell movements [33]. The Cls_{well}, a swelling-activated chloride channel, plays an important role in modulating the cell volume in articular chondrocytes [34]. Our data showed that the chronic radiation did not affect the gene expression of TRPM7 and Cl_{swell} in T lymphocytes,but that Cl_{swell} gene expression was markedly increased by lentinan. Nevertheless, it has been proven that ion channels play significant roles in T-cell activation, which in turn may modulate immune system activity [35].

The present study described the mRNA expression patterns of the six major ion channels in T lymphocytes isolated from mice under chronic radiation with or without lentinan treatment. Our results provide an additional reference for further research about the gene expression of these six ion channels in a mouse model of chronic radiation stress injury. More importantly, the present study revealed the mechanism of protection effects of lentinan in T lymphocytes against chronic radiation stress. We demonstrated that KV1.3 and Orai1 channel gene expressions were significantly influenced by radiation and ameliorated by lentinan.

There is a pitfall in our study. We performed mRNA analysis of the six ion channel genes, which does not always reflect the protein levels or functional changes. Thus, more research is needed to detect protein levels and activities of the ion channels in order to confirm the clinical significance of the present study.

In conclusion, we report here, for the first time, the impact of lentinan on the six major ion channels genes expression in the T cell extracted from mice under chronic radiation stress. The data suggest that Kv1.3 and Orai1 channel may mediate the anti-radiation effects of lentinan. Our results provide some novel information about the mechanism of protective effects of lentinan against chronic radiation stress.

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AUTHOR CONTRIBUTIONS

Theoretical analysis was carried out by M.-C. Li, and Q.-J. Fu. Experiments were carried out by Y. Wang, P.-P. Wang, J. Liu, and B. Zhang. All authors analysed and discussed the results. The manuscript was written by Y. Wang and P.-P. Lin. The figures were prepared by Y. Wang and Y.-H. Liu.

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УТИЦАЈ ЛЕНТИНАНА НА ГЕНСКУ ЕКСПРЕСИЈУ ГЛАВНИХ ЈОНСКИХ КАНАЛА У Т ЛИМФОЦИТИМА ИЗОЛОВАНИМ ИЗ МИШЕВА ИЗЛАГАНИМ ХРОНИЧНОМ ЗРАЧЕЊУ

Познато је да Kv1.3, KCa3.1, CRAC, TRPM7 и Cl_{swell} представљају пет значајних јонских канала у Т лимфоцитима. Да би се испитали механизми протективног деловања лентинана на оштећења Т лимфоцита до којих је дошло хроничним озрачивањем мишева, одређени су профили експресије Kv1.3, KCa3.1, Orai1, STIM1, TRPM7 и Cl_{swell} гена. Мишевима су, пре излагања ^{60}Co γ зрацима, убризгаване различите концентрације лентинана. Затим је из Т лимфоцита изолованих из јетре експерименталних животиња изолована укупна RNK. Метода квантитативне ланчане реакције полимеразе у реалном времену коришћена је за одређивање нивоа експресије Kv1.3, KCa3.1, Orai1, STIM1, TRPM7 и Cl_{swell} гена. Од шест јонских канала који су испитивани у овом раду, промена генске експресије у присуству лентинана код мишева изложених хроничном стресу зрачењем, запажа се само за Kv1.3 и Orai1. Експресија Kv1.3 и Orai1 била је значајно повећана у Т лимфоцитима који су изоловани из озрачених мишева, а третман лентинаном је враћа на контролни ниво. Наши резултати показују да се протективни ефекти лентинана можда остварују посредством Kv1.3 и Orai1 канала.

Кључне речи: лентинан, Т лимфоцит, Kv1.3, Orai1, хронични стрес зрачењем