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Evaluation of Potential Inflammatory Markers for Cystic Echinococcosis: P-selectin and Resistin

Kistik Ekinokokkoz için Potansiyel Enflamatuvar Belirteçlerin Değerlendirilmesi: P-selektin ve Resistin

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ABSTRACT

Objective: Cystic echinococcosis (CE) is one of the most common zoonotic diseases worldwide. Diagnosis of CE is predominantly based on imaging techniques and serological tests are used in cases of non-characteristic imaging findings as diagnostic reference. However, serological test results cannot be completely reliable as they are affected by multi-factors. P-selectin and resistin are inflammatory markers that are altered during the acute stages of infection. In this purpose, inflammatory markers as P-selectin and resistin have been investigated for a potential diagnostic reference for CE diagnosis.

Methods: A total of 60 patients who were diagnosed with CE and twenty-five healthy individuals were included in this study. Blood samples were obtained from all participants. Obtained sera were evaluated using the P-selectin and resistin ELISA kits for protein levels. Additionally, the relative expression of SELP (P-selectin) and RETN (resistin) genes were determined using the comparative CT (ΔΔCT) method between groups as CE patients with active and inactive cysts, CE patients and healthy controls.

Results: SELP (13.9-fold change, $p<0.05$) and RETN (8.1-fold change, $p<0.05$) were differentially expressed in CE patients compared in the control group. Whereas resistin protein levels were significantly higher in CE patients than the healthy controls ($p<0.001$), the difference in P-selectin protein levels was not significant ($p>0.05$). There was no difference between active and inactive CE patients in terms of P-selectin and resistin in gene and protein levels ($p>0.05$).

Conclusion: Although there was no difference between the active and inactive CE patients, the good differentiation between the healthy controls and the CE patients suggested that resistin is a potential inflammatory diagnostic reference

Keywords: Cystic echinococcosis, resistin, P-selectin, inflammatory marker, hydatid cyst

ÖZ

Amaç: Kistik ekinokokkoz (KE) tüm dünyada en sık görülen zoonotik enfeksiyonlardan biridir. KE tanısı çoğunlukla görüntüleme tekniklerine dayanmakta, karakteristik olmayan görüntüleme bulgularının olduğu durumlarda tanıya yardımcı olarak serolojik testler kullanılmaktadır. Ancak serolojik test sonuçları, birçok faktörden etkilendiği için tam anlamıyla güvenilir olamamaktadır. P-selektin ve resistin, enfeksiyonun akut evrelerinde değişen enflamatuvar belirteçler olarak bilinmektedir. Bu amaçla, KE teşhisi için potansiyel bir tanı referansı olarak P-selektin ve resistin gibi enflamatuvar belirteçler araştırılmıştır.

Yöntemler: Bu çalışmaya KE tanısı almış toplam 60 hasta ve 25 sağlıklı birey dahil edilmiş vetüm katılımcılardan kan örnekleri alınmıştır. Elde edilen serum, protein seviyeleri açısından P-selektin ve resistin ELISA kitleri kullanılarak değerlendirilmiştir. Ayrıca SELP (P-selektin) ve RETN (resistin) genlerinin göreceli ekspresyonu, aktif ve inaktif kisti olan KE hastaları ve sağlıklı kontroller arasında karşılaştırmalı CT (ΔΔCT) yöntemi kullanılarak belirlenmiştir.

Bulgular: SELP (13,9 kat, $p<0,05$) ve RETN'de (8,1 kat, $p<0,05$), kontrol grubuna kıyasla KE hastalarında farklı ekspresyon seviyeleri tespit edilmiştir. KE hastalarında resistin protein seviyeleri sağlıklı kontrollere göre anlamlı olarak yüksek iken ($p<0,001$), P-selektin protein seviyeleri arasındaki fark anlamlı bulunmamıştır ($p>0,05$). Aktif ve inaktif KE hastalarının P-selektin ve resistin açısından gen ve protein seviyesinde arasında fark bulunmamıştır ($p>0,05$).

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Türkiye PARAZİTOLOJİ Dergisi

Sonuç: Sonuç olarak, aktif ve inaktif KE hastalarının enflamatuvar belirteçlerinin gen ve protein seviyesinde arasında fark bulunmamasına rağmen, sağlıklı kontroller ve KE hastaları arasındaki ayrımın iyi olması nedeniyle, resistinin potansiyel enflamatuvar tanı referansı olabileceği gösterilmiştir.
Anahtar Kelimeler: Kistik ekinokokkoz, resistin, P-selektin, enflamatuvar belirteçler, kist hidatik

INTRODUCTION

Cystic Echinococcosis (CE) is one of the neglected diseases that affects around one million people worldwide. The causative agent of CE is larval form of *Echinococcus granulosus* sensu lato (s.l) (1). The infection always begins asymptomatically and clinical symptoms can vary from mild to severe. Although the liver is the most commonly affected organ for CE, it can be involved in every organ (2,3). Non-invasive imaging techniques especially ultrasonography (US) are the main tool for the diagnosis of CE. According to the World Health Organization-Informal Working Group on Echinococcosis (WHO-IWGE) classification, three clinical group have been identified as active (CE1 and CE2), transitional (CE3a/CE3b) and inactive (CE4 and CE5) (4). Until recently, surgery has been considered the only definitive therapy for liver CE, but new anthelmintic drugs and percutaneous interventional methods have extended the range of CE treatment. Even though there are a lot of options for treatment of CE, according to WHO-IWGE, the stage-specific approach should be implemented (2,3,5,6). For diagnosis, serological tests are used as supportive, however, the results can be deceptive due to variable sensitivity and specificity values (7). In addition, serodiagnosis can remain positive for years even after successful treatment (8). Therefore, serodiagnosis is not suitable to use for screening and confirm the presence of active infection.

Inflammatory markers can be used as biomarkers of active infection particularly for asymptomatic diseases (9). During inflammation, P-selectin (CD62P) is known as a mediator of leukocyte recruitment (10). P-selectins have a critical role in the parasite-induced (parasites of *Schistosoma* genus) progression of chronic liver disease (9). Resistin is a member of the resistin-like molecule (RELM) family of cysteine-rich secreted proteins that are present in humans (resistin and RELMb) and mice (11). Resistin is showed hormone-like activity at the beginning of inflammatory processes (12,13). Certain murine RELM proteins (RELMa and RELMb) are influenced by helminth infection (14). This study aims to determine protein and gene levels of P-selectin and resistin and to evaluate the potential of these inflammatory markers to be a diagnostic reference in the serum of CE patients.

METHODS

Ethical Considerations

The research protocol followed ethical guidelines of the Declaration of Helsinki. An informed consent that explained the purposes, benefits, and risks of the study was signed by the participants. This study was evaluated and accepted by the Hacettepe University Institutional Ethical Committee (no: 2020/16-72).

Sample Collection

A total 60 patients who were diagnosed with CE aged between 18-90 years after abdomen US examination were included in this study. All CE cysts were classified using the WHO-IWGE classification. In addition, twenty-five healthy individuals were included in this study after the US examination. Blood samples were taken from patients at the first diagnosis.

Serological Tests

All the collected sera were stored at -80 °C until study. The obtained serum samples were evaluated using the commercial serological test [Human P-selectin ELISA Kit (BT LAB, China) and Human Resistin ELISA Kit (BT LAB, China)] according to the manufacturer's instruction. All tests were performed in a same session.

RNA Extraction, cDNA Synthesis and Real-time-Polymerase Chain Reaction (RT-PCR)

Total RNA extraction was performed from serum of the patients and healthy controls using RNA Extracol (EURx, Poland) following the manufacturer's instructions. The RNA was quantified by FLUOstar Omega Microplate Reader (BMG LABTECH, Germany) using LVis plate. For cDNA synthesis, smART First Strand cDNA Synthesis kit (EURx, Poland) was used according to manufacturer's recommendations.

Expression levels of *SELP* (P-selectin) and *RETN* (resistin) genes were determined in the serum of CE patients and healthy controls. The primers for *SELP* and *RETN* genes were selected according to previous studies (15,16). RT-PCR were performed using SYBR Green Master Mix (A.B.T., Turkey) and primers at 200 nM final concentration with ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific). The relative expression of *SELP* and *RETN* genes were determined using comparative CT (ΔΔCT) method between CE patients and healthy controls. For normalization, *GAPDH* gene was used.

Statistical Analysis

GraphPad Prism 8.4.3 software was used for the statistical analyses. The results were interpreted with Mann-Whitney U test. The p-value under 0.05 was accepted as statistical significance.

RESULTS

Characteristics of Patients

Majority of the patients were female (63.3%, 38/60). The age range of the patients was between 18-85 years, and the mean age was 39.3. According to the WHO-IWGE classification system, 24 out of 80 CE cysts were identified as CE1, 14 as CE2, 3 as CE3a, 6 as CE3b, 28 as CE4 and 5 as CE5 respectively. Most of the patients had a single cyst (47/60, 78.3%), the rest harbored multiple CE cysts. No patient had a different type of hydatid cysts together. The average cyst diameter was recorded as 7.2 cm. The great majority of the CE cysts were involved in the liver (72/80, 90%), and also other organ involvements [spleen (2/80, 2.5%), kidney (3/80, 3.75%), bone (2/80, 2.5%) and intramuscular (1/80, 1.25%)] were recorded. All the patient's characteristics are presented in Table 1.

Expression Levels of *SELP* and *RETN* Genes

Comparative analysis was performed between 25 healthy control and 60 CE patients for the expression levels of *SELP* and *RETN*

Table 1. Characteristics of patients

Cyst type	Gender	Age	Cyst number	Cyst location	Cyst diameter (cm)
CE1	Female	18	1	Liver	5-10
CE1	Male	18	1	Liver	5-10
CE1	Female	49	1	Liver	5-10
CE1	Male	19	1	Liver	5-10
CE1	Male	32	1	Liver	<5
CE1	Female	39	1	Liver	>10
CE1	Male	21	1	Liver	<5
CE1	Female	24	1	Liver	5-10
CE1	Female	46	1	Liver	5-10
CE1	Female	25	1	Liver	>10
CE1	Male	21	Multipl	Liver	>10
CE1	Male	64	1	Liver	5-10
CE1	Male	61	1	Kidney	<5
CE1	Female	37	1	Liver	5-10
CE1	Female	21	1	Liver	>10
CE1	Male	18	1	Liver	>10
CE1	Male	82	1	Kidney	>10
CE1	Female	85	1	Liver	>10
CE1	Male	56	Multipl	Liver	>10
CE2	Male	41	1	Muscle	5-10
CE2	Female	54	1	Liver	>10
CE2	Male	54	1	Liver	5-10
CE2	Female	33	1	Liver	>10
CE2	Female	48	1	Bone	>10
CE2	Female	30	1	Liver	5-10
CE2	Female	39	Multipl	Liver	<5
CE2	Male	42	1	Liver	5-10
CE2	Male	46	1	Liver	<5
CE2	Female	31	1	Liver	>10
CE2	Male	17	1	Liver	5-10
CE3a	Female	35	1	Liver	5-10
CE3a	Male	26	1	Liver	5-10
CE3a	Female	26	1	Liver	<5
CE3b	Male	30	1	Liver	5-10
CE3b	Female	41	1	Liver	5-10
CE3b	Male	26	1	Kidney	5-10
CE3b	Female	18	Multipl	Liver	>10
CE3b	Female	60	1	Bone	<5
CE4	Female	22	1	Liver	5-10
CE4	Female	19	Multipl	Liver	<5
CE4	Female	78	1	Liver	5-10
CE4	Female	41	Multipl	Liver	<5
CE4	Female	32	1	Liver	5-10
CE4	Female	31	1	Liver	<5
CE4	Female	32	Multipl	Spleen	<5
CE4	Female	41	1	Liver	<5
CE4	Male	64	1	Liver	5-10
CE4	Female	33	Multipl	Liver	5-10
CE4	Female	41	1	Liver	5-10
CE4	Male	31	Multipl	Liver	5-10
CE4	Female	52	1	Liver	>10
CE4	Female	39	1	Liver	5-10
CE4	Female	30	Multipl	Liver	<5
CE4	Female	53	Multipl	Liver	5-10
CE4	Female	30	1	Liver	<5
CE4	Female	49	Multipl	Liver	5-10
CE5	Female	60	1	Liver	<5
CE5	Female	44	1	Liver	5-10
CE5	Male	44	Multipl	Liver	<5
CE5	Male	63	1	Liver	<5

genes using RT-PCR. *SELP* (13.9-fold change, $p<0.05$) and *RETN* (8.1-fold change, $p<0.05$) were differentially expressed in CE patients compared to control group (Figure 1). Additionally, *SELP* and *RETN* gene expressions were compared in patients with active (38/60) and inactive (22/60) cysts and there was no significant difference (Figure 2).

Levels of Protein Concentration

The levels of P-selectin and Resistin for CE patients and healthy controls were detected using ELISA and presented in Figure 3. Whereas resistin levels were significantly higher in CE patients than the healthy controls ($p<0.001$), the difference in P-selectin levels was not significant ($p>0.05$).

DISCUSSION

According to the WHO data, more than one million people are living with CE and alveolar echinococcosis (AE) worldwide at any one time and incidence data of human CE does not reflect real prevalence value due to the asymptomatic nature of the infection (17). According to the data of the Ministry of Health, while the morbidity rate was reported as 0.57 per 100,000 in 2008, it was reported as 2.25 in 2019 in Turkey (18). The most valuable epidemiological data for CE are obtained with active surveillance methods and screening using USG, unfortunately, these are both labor-intensive and costly. Hence, there is a need for new molecules that can be supportive markers for diagnosis.

Inflammatory markers have been studied in various diseases including helminth infections. Until now, several *ex vivo* studies on CE have demonstrated that the association between cytokine levels and disease progression (19,20). According to a previous report, compared to healthy individuals, levels of Interferon-γ (IFN-γ), Interleukin-12 (IL-12), IL-16, IL-18, IL-4, IL-5, IL-10, and IL-13 were found to be higher in the serum of CE patients (21). Among the inflammatory markers, P-selectin and resistin has been evaluated in some parasitic infections (9). However,

there is lack of data on their role in CE. In this study, P-selectin and resistin expression were investigated at both gene and protein levels in CE patients compared with healthy controls.

P-selectin (CD62P) is among the immune system components and can play a role as a mediator in leukocyte recruitment during inflammation (22). Besides, P-selectin is an adhesion molecule that migrates to the surface of endothelial cells and platelets under inflammatory stimuli (23). Platelets are among the immune system components and can play a role as mediator cells in inflammation via potent pro-inflammatory substances (24,25). Platelets are involved in the protection against helminths as potent effector cells (26). A previous report showed that CD62P expression was significantly upregulated in CE patients compared to the control group, suggesting the presence of platelet activation during CE infection (27).

In this study, we have confirmed that SELP gene expression (13.9-fold change, $p<0.05$) were found to be significantly up-regulated in the CE patients compared to healthy controls. In other studies, it is reported that the correlation between gene expression measured at the mRNA level and the corresponding protein level

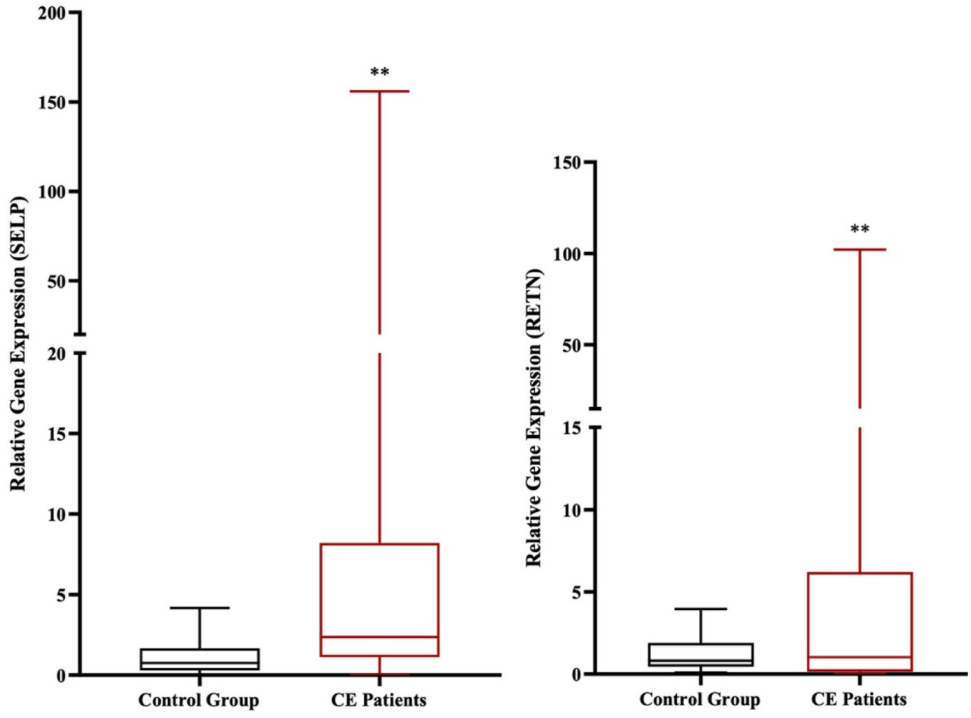

Figure 1. Relative gene expressions between CE patients and healthy control group. Relative *SELP* gene expression (13.9-fold change, $p<0.05$), relative *RETN* gene expression (8.1-fold change, $p<0.05$)

CE: Cystic echinococcosis

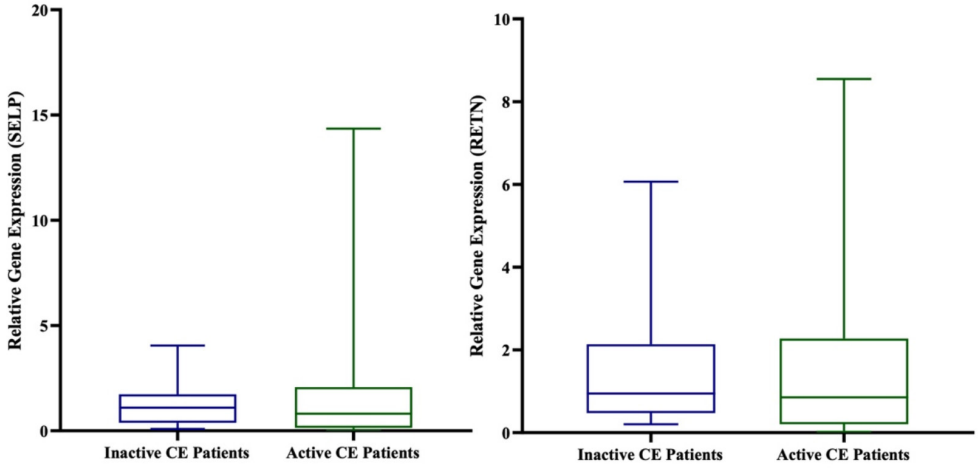

Figure 2. Relative gene expressions between active and inactive CE patients, relative *SELP* gene expression ($p>0.05$), relative *RETN* gene expression ($p>0.05$)

CE: Cystic echinococcosis

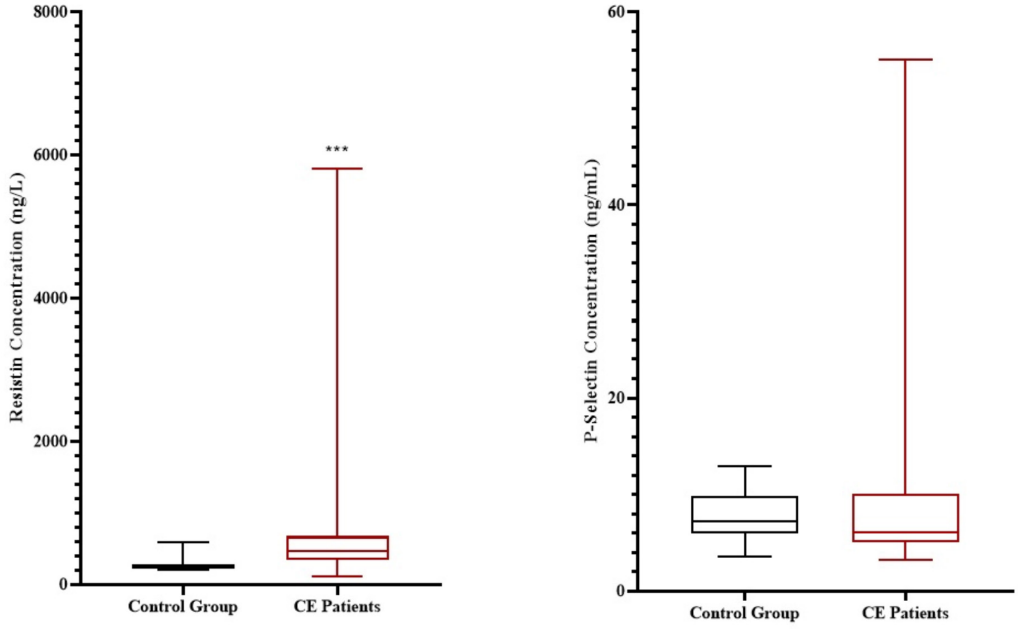

Figure 3. The levels of resistin concentration for CE patients and healthy controls ($p<0.05$), the levels of P-selectin concentration for CE patients and healthy controls ($p>0.05$)

CE: Cystic echinococcosis

is generally weak (28-30). Thus, protein levels of P-selectin were also investigated to observe the correlation between the mRNA expressions and protein levels. However, there was no statistically significant difference in protein levels of P-selectin between CE patients and healthy controls.

On the other hand, biological effects of human resistin have been investigated in helminth and viral infections though its exact function in humans is still unclear. Based on previous studies, plasma resistin levels were up-regulated in both chronic filarial nematode infection and soil-transmitted helminth infection (14). In addition, it was found to be correlated with increased parasite burden and elevated levels of inflammatory cytokines TNFα, CCL2 and IL-6 (31). According to our results, compared to healthy controls relative RETN expression was significantly higher in CE patients. Besides, protein levels of resistin were significantly elevated in CE patients, as well. Previous reports indicated that resistin expression was increased in macrophages from patients with filarial infection following exposure to parasite antigen (32,33). In addition, a lot of studies have demonstrated that lipopolysaccharide (component of Gram-negative bacteria) can influence resistin expression *in vivo* and *in vitro*. However, accumulating data has shown that resistin expression is a part of innate immune response to various helminth infection. Hence, resistin may play different roles in two types of immune responses as type 1 inflammatory process triggered by bacterial components and Th2 based immune response induced by helminths (14).

We have also performed the comparison of P-selectin and resistin expression levels in patients with active and inactive cysts and found no difference between the two groups of CE patients. Although there was no difference between the active and inactive patients, the good differentiation between the healthy controls and the CE patients suggested that they may be potential inflammatory diagnostic references.

CONCLUSION

P-selectin and resistin serum levels have been comparatively evaluated between CE patients and healthy controls for the first time. In addition, the present study has demonstrated that resistin is a potential inflammatory marker for CE diagnosis. More investigations characterizing the infection on the inflammatory pathways should be conducted, especially for the asymptomatic people with poor access to healthcare groups.

*Ethics

Ethics Committee Approval: The research protocol followed ethical guidelines of the Declaration of Helsinki. This study was evaluated and accepted by the Hacettepe University Institutional Ethical Committee (no: 2020/16-72).

Informed Consent: Informed consent that explained the purposes, benefits, and risks of the study was signed by the participants.

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*Authorship Contributions

Surgical and Medical Practices: T.Ç., E.Ü., A.B.D., D.A., O.A., Concept: S.Ö., T.Ç., E.Ü., A.B.D., D.A., Y.A., O.A., Design: S.Ö., S.Y.Ç., Y.A., O.A., Data Collection or Processing: S.Ö., İ.B., Analysis or Interpretation: S.Ö., İ.B., S.Y.Ç., Literature Search: S.Ö., İ.B., Writing: S.Ö., İ.B., S.Y.Ç., Y.A., O.A.

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