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Derleme/Review

Molecular Applications fortheDiagnosis of *Echinococcus granulosus* Infection and New Approaches

Echinococcus granulosus İnfeksiyonu Tanısında Moleküler Uygulamalar ve Yeni Yaklaşımlar

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ÖZET

Kistikekinokokkozis (KE), tüm dünyaya yayılan, büyük bir hastalık yüküne neden olan ve ara konaklarda uzun süreli hidatik kist büyümesi ile karakterize, kronik zoonotik bir hastalıktır. KE etkeni olan *Echinococcusgranulosus*, çoğunlukla karaciğerde (%65-70) ve akciğerlerde (%20-25) ve diğer organlarda (%2 böbrek, %2 dalak ve %2'den az beyin vb.) hidatik kistlere neden olmaktadır. KE tanısı klinik bulgulara, görüntüleme yöntemlerine, serolojik ve moleküler tekniklere dayanır. Hasta serumundaki *Echinococcus* DNA'sının belirlenmesi tanıda invaziv olmayan uygulanabilir bir yöntem olabilir. Şimdiye kadar, farklı *E. granulosus*genotipleri, insanlardan ve diğer ara konaklardan moleküler teknikler kullanılarak tanımlanmıştır. Ama şimdi moleküler yaklaşımlar sadece DNA seviyeleriyle değil, aynı zamanda RNA seviyeleriyle de sınırlıdır. Özellikle genomik, proteomik, mikrodizi ve yeni nesil dizileme analizlerindeki yeni gelişmeler, tanı, aşılama ve kemoterapi için ek hedeflerin belirlenmesinde faydalı olacaktır. Yüksek çıktılı analiz yöntemlerinin kullanılması, *E. granulosus* ile konakları arasındaki etkileşim mekanizmasının temelini oluşturmaya yardımcı olabilir. Böylece, elde edilen yeni bilgiler, *E. granulosus* enfeksiyonunun yeni tedavi ve tanısal hedeflerini geliştirmek için kullanılabilecektir.

Anahtar Kelimeler: E. *granulosus*, KistikEkinokokkozis, Moleküler Teknikler, DNA, RNA.

ABSTRACT

Cystic echinococcosis (CE) is a chronic zoonotic disease which is distributed all over the world, causes a large disease burden, and characterized by prolonged growth of hydatid cysts in intermediatehosts. *Echinococcus granulosus* which is a CE agent and causes hydatid cysts in mostly in liver (65-70%) and lungs (20-25%) but also other organs (kidney 2%, spleen 2% and brain less than 2%, etc.). The diagnosis of CE is based on clinical findings, imaging techniques, serological and molecular technics. Identification of *Echinococcus* DNA in patient serum may be an applicable non-invasive method in the diagnosis. Up to now, different genotypes of E. granulosus have been identified by using molecular techniques from humans and other intermediate hosts. But now, the molecular approaches are not restricted to DNA levels but also to RNA levels. Especially new developments in genomics, proteomics, microarray, and next generation sequencing analysis will be useful for the identification of additional targets for diagnosis, vaccination, and chemotherapy Using high through put analysis methodologies can help to underly the mechanism of interaction between *E. granulosus* and its hosts. So, obtained new informations will be used to develop new therapeutic and diagnostic targets of *E. granulosus* infection.

Keywords: *E. granulosus*, Cystic Echinococcosis, Molecular Technics, DNA, RNA

INTRODUCTION

Cystic echinococcosis (CE) is a zoonotic disease distributed worldwide, causing an enormous disease burden, and characterized by prolonged growth of hydatid cysts in intermediate hosts. *Echinococcus granulosus*is a CE agent. Adult *E. granulosus*develops in the herbivores such as dogs, foxes, wolves which are the definitive hosts of *E. granulosus* and locates in the small intestine. Herbivores and humans (which is accidental) are intermediate hosts. CE typically affects the liver and lungs by accidental ingestion of eggs of the

E.granulosus. The larval stage of tape worm infects humans, and hydatid cysts exist mostly in the liver (65-70%) and lungs (20-25%) but also other organs (kidney 2%, spleen 2%, and brainless than 2%) (Altintas, 2003; McManus et al., 2003; Moro and Schantz, 2009). Several *E. granulosus* genotypes are recognized in which some of them have distinct intermediate host preferences. However, not all genotypes cause infections in humans. The genotype causing CE infections in humans are sustained in a dog–sheep–dog cycle (Thys et al., 2019).

Cystic echinococcosis is a significant helminthic disease in Turkey, presenting a public health and economic problem because most people live in upstate and are dealing with husbandry (Altintas, 2003). Ministry of Health reported 408 annual cases in 2008, and the number is increasedto 1,702 by the end of 2019. The morbidity was reported as 0.57 per 100.000 in 2008 and as 2.08 in 2019 (Altintas et al., 2020).

Cystic echinococcosis is characterized by the long-term growth of hydatid cysts filled with hydatid cyst fluid (HCF) and protoscoleces in humans. The diameters of cysts generally increase 1-5 cm each year. CE can be represented by a wide range of clinical signs that depend on the cyst's location. The cysts' growth rates may vary depending on being in the same organ or within the same individual and between individuals invarious regions (Altintas et al., 2020).

CE's annual global costisestimatedtobemore than $750 million for human infection and more than $ 2 billion for livestock infection (WHO, 2017).

Diagnosis

CE diagnosis is based on clinical findings, imaging techniques, and serology. The presence of protoscoleces can be shown by microscopic examination of the fluid (Brunetti et al., 2010).

Many factors including the numberand stage of the cysts and the cyst's location are used in CE prognosis. Therefore, the management of the disease is very complicated. Radiography, ultrasonography (US), computerized tomography (CT), and magnetic resonance imaging (MRI) could be useful diagnostic methods for human CE. Ultrasonography is a useful technique to diagnose both CE and alveolar echinococcosis (AE) and should be validated by CT and MRI. Besides, it is the basis of CE diagnosis in abdominal locations (Macpherson and Milner, 2003). The WHO echinococcosis expert group has established an international classification of ultrasound images of the CE, which in principle should be used when a US diagnosis is made. Laboratory diagnosis for human CE is primarily serological tests. The current gold standard serodiagnosis is based on detecting IgG antibodies against native or recombinant antigen B subunits derived from cyst fluid in Enzyme-Linked Immunosorbent Assay (ELISA) or in immunoblots (Craig et al., 2007). Ancillary methods are mainly based on the detection of serum antibodies against HCF but rise to false-positive results obtained depending on cross-reactive antigens ofHCF. In addition, some patients can be tested negative inspite of suffering from the disease. Now with the new recombinant techniques can define various recombinant antigens derived from *E. granulosus*. The combination of several methodologies, including antibody- antigen detection and recombinant antigens, could give rise to the performance of the adjunctive laboratory methods, enabling In-depth understanding ofhost-parasite relationships and parasite phenotype at different developmentalstages to get the best diagnostic tool and make it available to use it in clinical practice.

Molecular Epidemiology

Echinococcus granulosus is the major zoonosis forcystic echinococcosis, and there is growing evidence that they are excellent models for studying host-parasite cross-talk between two mammalian hosts. It is essential to understand the biology of parasites to enlighten the mechanisms involved in how the parasite causes chronic disease. Dif-

ferent genotypes of *E. granulosus*have been identified by using molecular techniques (sequencing, phylogenetic analysis) from humans, sheep, camel, etc. (Macpherson and Milner, 2003; Altintas et al., 2013; Zhang et al., 2014). Nevertheless, now, the molecular approaches are not restricted to DNA levels but also RNAlevels.

Approaches on DNA Level

PCR based methods

Diagnosis of CE in the early-stage is crucial for effective drug treatment, but CE is usually detected at the last stage when the cystis large andcomplex. Therefore only therapeutic option ismostly surgery. So, in the diagnosisof CE, *Echinococcus* DNA detection in patient serum could be used as a non-invasive method. For this purpose, cell-free DNA (cfDNA) detection has been a powerful tool for definitive diagnosis. Cell-free DNA composed of nucleic acid fragments is widely used in various clinical settings such as tumor monitoring, non-invasive prenatal testing (NIPT), etc. (Jiang et al., 2016). To date, many parasitic diseases such as*Leishmania* (Calderon et al., 2011), *Plasmodium* (Ghayour Najafabadi et al., 2014), *Schistosoma* (Wichmann et al., 2009), *Trypanosoma* (Russomando et al., 1992), and *Wuchereria*spp. (Ximenes et al., 2014) have been successfully detected with cfDNA. Cell-free DNA of *Echinococcus* spp was previously proposed as a biomarker for echinococcosis, and cfDNA was found to exist in plasma or serum with PCR-based methods (Gottstein et al., 2014). Generally, PCR, qPCR, or Loop-Mediated Isothermal Amplification (LAMP) used for *Echinococcus* genotyping (Salant et al., 2012; Boubaker et al., 2017). Several studies confirm that PCR can be useful on serum samples but not in urine samples to confirm the parasitic diagnosis and has advantages in rapid diagnosis and large-scale epidemiological research (Chaya et al., 2014; Shang et al., 2019). Assays based on detecting circulating antigens from *Echinococcus* such as immuno-PCR and latex agglutinationtest (LAT), were reported to have high specificity (Zhang et al., 2012; Mirzapour et al., 2020).

DNA sequencing

Sanger sequencing enables selective inclusion of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DN Areplication and is used to determine the DNA nucleotide sequence. Four species and ten genotypes are classified in *E. granulosus* sensu lato depending on their host range and genetic diversity of which *E. granulosus* sensu stricto (G1 to G3), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5), and *Echinococcus canadensis* (G6 to G10) (Ancarola et al., 2017). Mitochondrial DNA (mtDNA) markers are ideal for evolutionary studies such as phylogeography, population genetics, phylogeny, etc. Therefore, for the analysis of the mitochondrial genetic structure in CE genotypes, mitochondrial cytochrome c oxidase I (cox1) and NADH dehydrogenase subunit I (nad1) genes were also evaluated in the serum of CE to determine the source of DNA and compared to cyst tissue samples (Altintas et al., 2013; Jafari et al., 2018; Moradi et al., 2019; Santucciu et al., 2019; Altintas et al., 2020).

Next-Generation Sequencing (NGS)

Next-Generation Sequencing (NGS) is a new technology enabling sequencing a genome quickly. All NGS platforms can perform parallel sequencing of millions of small DNA fragments. Bioinformatic analyses are used to combine all these parts by mapping the individual reads to the reference genome. Each base in the genome is sequenced multiple times and provides high depth readings to present accurate data. Although the NGS technique provides a new method for comprehensive screening and appropriate echinococcosis diagnosis with high specificity, this is a new area for detecting *Echinococcus* DNA. Recently it has been shown that the repeat region targeted sequencing was a precise detection of *Echinococcus* infection (Wan et al., 2020).

Approaches on RNA Level

Non-coding RNAs, which are essential gene regulators on post-transcriptional levels, became hot topics for analyzing the critical mechanisms of *E. granulosus*. The mechanisms underlying the involvement of different development stages, including miRNAs, remain unknown. To better understand the parasite-host interplay in *Echinococcus* infections, new genomics, proteomics, microarray, and NGS analysis will help identify additional targets for diagnosis, vaccination, and chemotherapy (Cucher et al., 2011; Macchiaroli et al., 2015).

Microarraytechnology

Microarray technology is used to identify and characterize gene expression profiles, enabling the analysis of a whole genome's differentially expressed genes in one experiment. Therefore, microarray technology has become widely used to determine whether human miRNAs and lncRNAs are expressed differently during *E. granulosus* infection. Besides, cDNA microarray methods were used to detect gene expression profiles of *E. granulosus* in order to understand the pharmacological mechanism of anti-echinococcosis drugs (Lu et al., 2014).

Almost 10–14% of the *Echinococcus* genome comprises protein-coding genes and theremains are transcribed as non-coding RNAs (Tsai et al., 2013). Non-coding RNAs are classified into two main groups. miRNAs are in the small non-coding RNA class of 19–24 nucleotides in length, regulating the gene expression post-transcriptionally by inhibiting protein translation or target transcripts by binding with their seed sequences (Kim et al., 2009). In contrast, lncRNAs are longer than 200 nt, and they have lower expression than protein-coding genes (Huang et al., 2018). MiRNAs and lncRNAs are widely expressed in *Echinococcus* spp. (Ancarola et al., 2017; Yu et al., 2018). Recent research has shown that circulating miRNAsof both parasite and host origin can be detected in humans and animals' blood or helminth infectious fluids (Cai et al., 2016). Therefore, they are potential diagnostic biomarkers for the early diagnosis. In a study suggesting that host miRNAs are involved in the human-parasite interactionof *E. granulosus*, eight miRNAs were found to be up regulated (let-7g-5p, let-7a-5p, miR-26a-5p, miR-26b-5p, miR-195-5p, miR-16-5p, miR-30c-5p, and miR-223-3p) and associated with the presence of functional cysts (Mariconti et al., 2019). Besides, in the subcutaneous adipose tissues from mice infected with *E. granulosus* protoscoleces, 1052 mRNA and 220 lncRNA transcripts were found to be differentially expressed (Lu et al., 2020).

CircularRNAs (circRNA), which are an other type of non-coding RNA, are endogenous RNAs without 5 ′ end caps or 3 ′ poly(A) tails and are expressed in tissue-specific and developmental stage-specific models (Kalifu et al., 2021).

Transcriptome Profiling

RNA-Seqisatechnique that uses NGS to analyze the entire transcript to me to reveal the presence and amount of RNA in a biological sample. This technology can be used to explore the diversity and expression patterns of *E. granulosus* miRNAs in different life stages. Using high throughput technologies is the new hot-spots to evaluate the small RNA composition and miRNA expression changes during *E. granulosus* development. With this technology, not also mature miRNAs but also novel miRNAs can be detected. With this respect, recent studies have characterized the miRNAs in three developmental stages of *E. granulosus*, and 114 mature miRNAs and 62 miRNA stars have been evaluated (Bai et al., 2014). The prevalence of alternative splicing using NGS in protoscolex transcriptomes of *E. granulosus* and *E. multilocularis* was found to be approximately 33-36% (Liu et al., 2017) whereas 109 known miRNAs and 189 novel miRNA hairpin precursors were detected (Wang et al., 2018). *E. granulosus* was also used as a model to study the molecular basis of the-host-parasite cross-talk during cestode infections.

Moreover, it provides a data for the gene expressions involved in essential aspects of *E. granulosus* biology, such as metabolism and the synthesis of crucial parasite structures (Moro and Schantz, 2009; Pan et al., 2014). A comprehensive characterization of the *E. granulosus* transcriptome or proteome will provide valuable information on *Echinococcus* biology and the interplay between the parasite and its definite or intermediate hosts. Analysis of the *E. granulosus* adult worm proteome was contributed to the literature as the first report in proteomic studies (Cui et al., 2013). Such studies reveal antigenic profiles and expression characteristics of *Echinococcus* and give insight into evasion mechanisms during infection and echinococcosis immunopathology.

Different cell types release extracellular vesicles (EVs) that have a membranous origin. They are mainly found in specific vesicles known as exosomes. EVs, containing exosomes can carry developmental signal proteins that regulate the growth and formation of various parasites and contain proteins, carbohydrates, lipids, microRNAs, and other small RNAs (Schorey et al., 2015). To identify exosomal cargo content, EVs are isolated from HCF. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is used to quantify and visualize proteins in the exosomal fraction, RNA-seq is used to identify transcriptome profiling of exosomal RNA. It has been shown that the exosome-like vesicles (ELVs) of parasites can transfer non-coding RNAs to host cells to regulate gene expression; however, the ncRNAs contents of the ELVs from *E. granulosus*are unknown. Therefore exosome studies provided essential resources for further analysis of potential ncRNAs in exosome-like vesicles. Determination of exosome cargo content will allow new markers to be discovered to diagnose and prevent CE (Zhang et al., 2020).

Conclusion

Using high-through put analysis methodologies can help lay the foundation for the interaction mechanism between *E. granulosus* and its hosts. These informations could be useful for developing new diagnostic and therapeutic targets for cystic echinococcosis

Conflict of interest

There is no conflict of interest.

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