In the name of God

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Title:

Determination of Changing Expression of miR-212 and EGFR Genes in Clinical Samples of Trichophyton rubrum Dermatophyte in Infected Sites compared to Adjacent Healthy Sites

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Abstract

Introduction & Goals: Skin infections with dermatophytes are common in the world and are called Tinea. As an anthropophilic fungus, Trichophyton rubrum is the most frequent cause of Tinea in the world. Antimicrobial peptides (AMPs) such as HBD-3 and RNase7 are effective molecules in innate immunity of the skin, which have potential antimicrobial effect,rapidly kill microorganismsand are affected by EGFR gene,the increasing expression of which in skin cells activates them and prevents the colonization of organisms, including dermatophytes in keratinocytes. However, through increasing expression of microRNAs, and in particular miR-212 in this study, the mRNA of EGFR gene is silenced and colonization of the dermatophyte in the skin leads to dermatophytosis. The aim of this study was to determine the changes in the expressions of miR-212 and EGFR genes in clinical specimens infected with Trichophyton rubrum dermatophyte in infected sites compared to adjacent healthy sites.

Methods: Collection of clinical specimens was based on 36 samples of tissue infected with Trichophyton rubrum, collection of 36 control samples from the margin of the fungus-infected tissues in the same patients, extraction of whole RNA and its optimization, synthesis and optimization of cDNA, amplification of EGFR and miR-212 genes by Real-Time PCR (SYBER green), data collection and analysis was performed.

Results: After culture of skin flakes on Mycosel agar medium and the appearance of Trichophyton rubrum colonies, a slide culture was prepared from the colonies to examine the reproductive organs of the fungus and the presence of T. rubrum was confirmed by 40x microscopy. After extracting whole cellular RNA from each of the clinical samples, the concentration of each sample was determined with Nanodrop device. The values obtained for the samples ranged 1.7-1.9 by the device, which indicate the acceptable purity of the extracted RNA. Subsequently, to determine the quality of RNA and maintain its integrity, the RNA extracted from each sample was run on 1% agarose gel. A gentle, uniform smear was thoroughlyexamined from top to bottom of the gel, and 28S, 18S, and 5S ribosomal RNA bands were clearly visible in each sample. Finally, Real-TimePCR was performed on both genes along with internal control genes (GAPDH and miR-103a-3p) and the resulting curves are shown in the fourth chapter.

Conclusion: Bioinformatics analysis showed that miR-212 can affect EGFR as a potential target; however, functional studies are needed to demonstrate it. MiR-212 in tissue samples infected with Trichophyton rubrum dermatophyte significantly reduced the expression of EGFR gene, and the expression of miR-212 gene increased by about eight fold compared to that of EGFR gene. On the other hand, in tissue samples used as control, the expression of miR-212 was much less1 than that of EGFR gene, so the subject has been able to resist the invasion of T. rubrum in the peripheral areas of the lesion due to the presence of EGFR gene and consequent presence of AMPs.

Keywords: Tinea - Trichophyton rubrum - Gene Expression - AMP - EGFR - Real-Time PCR - miR-212

CHAPTER I

INTRODUCTION

1-1- Introduction and importance of the subject

Dermatophytosis is one of the most common infections in the world, which is known as Tinea (1, 2). These infections include a wide range of diseases which are highly prevalent but not fatal. Dermatophytosis affects the skin and its appendages (hair and nails), is mainly limited to the keratin layers of the skin and stratum corneum of the epidermis and is caused by various pathological changes in the host as well as because of the presence of fungal agents and their metabolic products. The cell wall of dermatophytes is mainly composed of chitin, glucan and glycopeptides, which are important antigens of the dermatophyte. Trichophytin is indeed an antigenic extract of dermatophytes, especially in Trichophyton species. Most dermatophytes grow best in the presence of one or more amino acids or a number of proteins. Several dermatophytes are autotrophic but certain species are unable to produce certain types of vitamins and are in need of these factors for their growth and development, a feature that is useful for the diagnosis of several types of Trichophyton. These fungi have two stages in their life cycle. Anamorphic or asexual and incomplete stage, namely the stage of fungi when they are isolated in the laboratory and the other stage is teleomorphic (sexual and complete stage), although not all dermatophytes have a sexual stage (3). Dermatophytes cause infections in the skin, hair and nails due to their keratinophilic nature and they include three genera: Microsporum, Epidermophyton and Trichophyton (4).

The sexual stage of Trichophyton and Microsporum is called arthroderma and nannizzia, respectively, but no specific sexual stage has been reported for Epidermophytons. Ecologically, these species are anthropophilic, zoophilic and geophilic, are highly related in terms of physiological and antigenic properties and are able to cause a variety of clinical symptoms in different anatomical sites, includingT. copitis, T. corporis,T. unguium,T. cruris, T. pedis, T. faciei, T. manum and T. barbae (3).

The pathogenesis of Tinea is not fully understood, but a hallmark of dermatophytes is that they have the ability to inhabit the skin and release enzymes that break down the host fat and protein. Specifically, dermatophytes are able to break down creatine and use it to produce nitrogen, which leads to their colonization on the skin and penetration into the host epidermis (5). Dermatophytosis is dependent upon several factors such as migration, environmental conditions, lifestyle, age, the presence of diseases and the immune system (6). Tinea is the only fungal disease that is actually transmissible through the skin and the majority of dermatophytes (80%) do not have a saprophytic form in nature and are completely pathogenic.Nearly 50% of Tinea cases are caused by anthropophilic dermatophytes and the rest by zoophilic and geophilic dermatophytes. Reports of Tinea date back to 14th century and are today of particular importance in both underdeveloped and developed countries (3).
Most studies on dermatophytosis have been conducted in Greece (7-10). In a five-year study (2011-2015) on 2910 patients admitted to teaching hospitals in Greece, out of 2751 patients diagnosed with clinical symptoms of dermatophytes, 294 (10.1%) were infected with dermatophytes through direct microscopic tests (slide preparation) and culture of samples. The highest infection rate was related to Microsporum canis followed by Trichophyton rubrum (35.1%) (11), (Table1-1).

Table 1-1: Dermatophyte genera identified in Greece (2011-15) (11)

Species	NO.	Percentage (%)
Microsporum canis	100	35.8
Trichophyton rubrum	95	35.1
Trichophyton mentagrophytes	65	23.3
Epidermophyton floccosum	7	2.5
Microsporum gypseum	5	1.6
Trichophyton violaceum	2	0.7
Trichophyton verrucosum	1	0.4
Trichophyton tonsurans	1	0.4
Total	279	100

As can be seen in (Table 1-2), T. corporis has been the most common form of dermatophytosis, although the most frequent site of T. rubrum involvement is reported to be nail, foot and groin, causing T. unguium, T. pedis

and T. cruris, respectively (8).

Table 2-1: The ratio of dermatophytes incidence in relation to the sex of the person with Tinea (male-female) and the site of fungal infection (11)

Dermatophyte	Tinea Corporis M/F	Tinea Unguium M/F	Tinea Pedis M/F	Tinea Capitis M/F	Tinea Faciei M/F	Tinea Cruris M/F	Tinea Manuum M/F
Microsporum canis	17/33	0/2	0/1	19/14	8/5		0/1
Trichophyton rubrum	14/5	22/17	15/11		2/3	4/2	2/1
Trichophyton mentagrophytes	12/10	7/6	8/9	1/1	3/2	1/2	2/1
Epidermophyton floccosum	1/0	0/1	2/2			1/0	
Microsporum gypseum	0/3				0/1		1/0
Trichophyton violaceum		1/0				0/1	
Trichophyton verrucosum							1/0
Trichophyton tonsurans	1/0						
Total	45/51	30/26	25/23	20/15	13/11	6/5	6/3

The results of investigations conducted over the mentioned years on age and type of Tinea caused by dermatophytes are shown in (Table 1-3) and (Table 1-4).

Table 3-1: Different age groups with Tinea in various parts of the body (11)

Tinea	<15y	15-30y	31-45y	46-60y	>60y
Tinea Corporis	26.1	20.8	22.9	15.6	14.6
Tinea Unguium	0	5.4	32.1	37.5	25
Tinea Pedis	0	10.7	35.4	31.3	22.9
Tinea Capitis	94.2	0	2.9	0	2.9
Tinea Faciei	45.8	16.7	4.1	16.7	16.7
Tinea Cruris	9.1	27.3	27.2	27.3	9.1
Tinea Manuum	22.2	33.4	11.1	11.1	22.2

Table 4-1: Different age groups with Tinea and its causative agents (11)

Species	<15y	15-30y	31-45y	46-60y	>60y
Microsporum canis	61	11	13	11	4
Trichophyton rubrum	2	13.3	30.6	28.6	25.5
Trichophyton mentagrophytes	13.80	13.8	30.6	23.1	18.5
Epidermophyton floccosum	0	0	0	28.6	71.4
Microsporum gypseum	0	20	0	60	20
Trichophyton violaceum	0	100	0	0	0
Trichophyton verrucosum	0	100	0	0	0
Trichophyton tonsurans	0	100	0	0	0

Epidemiological studies show that from 1992 to 2010, T. rubrum had been the most common cause of dermatophytosis (8, 9, 12). Studies in 20 European countries have found that T. pedis(athlete's foot) and T. unguium are the most prevalent diagnosed fungal infections (13).

However, according to epidemiological studies in several countries, it has been found that T. mentagrophytes is the most common cause of Tinea in Iran(10, 14-19) and that in T. pedis the lowest rate of infection was related to T. mentagrophytes and T. rubrum(8-10, 13, 19). Since our research concerns T. rubrum, we will focus only on this fungus and describe the extent of its involvement, its place in the family of fungi, its morphology and treatment. The highest infection rate with T. rubrum in Iran is mentioned in winter with low temperature and high humidity, while the season of the year with highest incidence of this fungus has been reported insummer in other countries (20),(Diagram 1-1).

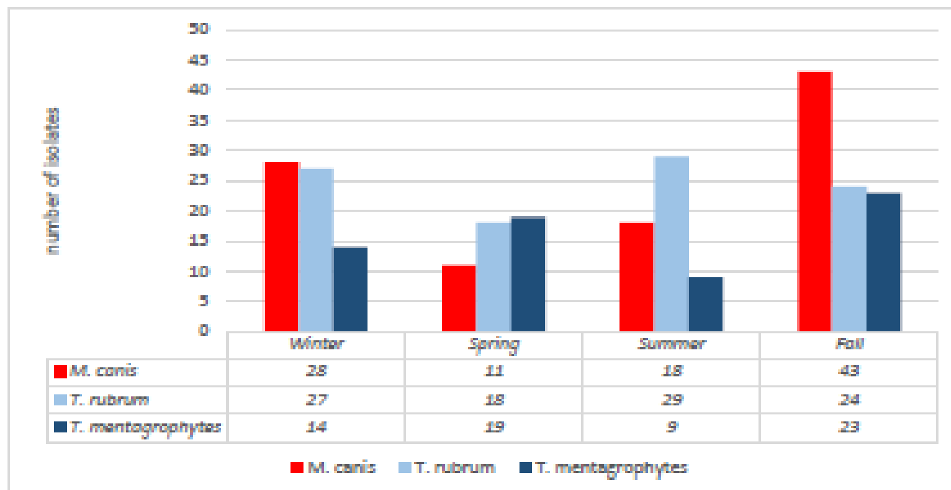


Diagram 1-1: Incidence of dermatophytosis in different seasons of the year (11)

2-1- Trichophyton rubrum

1-2-1- Pathogenesis of Trichophyton rubrum

Given that T. rubrum is an anthropophilic fungus, it is less common in animals (21). However, Van Brozgem believes that an experimental infection by T. rubrum could be developed in guinea pigs, and Rice was able to cause the disease in a rabbit irradiated on the abdomen(3).
Infection with T. rubrum is more common in men than in women. Although infection with the fungus is more prevalent in adults, there have been reports of infection in children as shown in, (Table1-3) (22).
The fungal infection is both acute and chronic and can last for a lifetimein some people. The infection may subside for a while but recur frequently over time. If the antidermatophyte activity of serum is low,the fungus will extensively invade the epithelial cells and dermis (23).

2-2-1- Taxonomy of Trichophyton rubrum

Specific classification of T. rubrum is as follows (24).

- Kingdom: Fungi
- Phylum: Ascomycota
- Subphylum: Pezizomycota
- Class: Eurotiomycotina
- Order: Onygenales
- Family: Arthrodermataceae
- Genus: Trichophyton
- Species: **Trichophyton rubrum**

Trichophyton megninii, Trichophyton fischeri, Trichophyton raubitschekii, and Trichophyton kanei are other names of this fungus, which was discovered and described by Pehr Henrik Malmsten in 1845 (25-27).

3-2-1- Characteristics of Trichophyton rubrum

If the fungus attacks the hair, the infection is of ectothrix type, is urease negative and does not perforate the hair but absorbs the sorbitol, showing red and light green pigment on Corn meal Agar and Littman Oxgall agar,respectively. T. rubrum lacks fluorescence and its sexual stage is not yet known.

4-2-1- Morphology of Trichophyton rubrum colony

In terms of morphology and appearance of colony, the fungus grows slowly on Sabouraud dextrose agar medium with cyclohexamide, chloramphenicol and gentamicin without pH change and forms white cottony colonies having red underside. If the culture stays longer, it will turn brown. Using a number of compounds, the fungus consumes the glucose of the culture medium, which sometimes produces no red color. The reason for the development of red color is the storage of compounds released during the use of casamino acids erythritol albumin agar (CEA) (21).

5-2-1- Microscopic view of Trichophyton rubrum

In microscopic view, the mycelia are colorless, transparent with transverse walls. The microconidia are teardrop-shaped or nail-shaped, which are scattered around the mycelia. The macroconidia have smooth, thin and parallel walls that are pencil-shaped and long with 2-8 middle blades.Although there a large number of macroconidia in the specimens isolated from inflamed lesions, they are lost during isolation of the fungus. The types isolated from chronic lesions usually lack microconidia or macroconidia. Chlamydoconidia and racquet-shaped hyphae are also seen when the nutrients in medium are decreased (21), (Figure 1-1).

Figure 1-1: Microscopic view of Trichophyton rubrum

6-2-1- Treatment of Tinea caused by Trichophyton rubrum

Topical and oral medications are used for this purpose as follows:

A) Topical drugs such as Tinaderm ointment and Mycodecylointments,

B) Oral drugs like griseofulvin at a dose of 12.5 mg per kilogram of body weight every other day, which is generally effective on fungi causingTinea. Obviously, despite the usefulness of griseofulvin forT. rubrum lesions, it takes 4-6 weeks for all clinical signs to disappear. Other antifungal drugs include Tolnaftate or miconazole, econazole or clotrimazole, and the like (3).

3-1- Pathogenesis mechanism of dermatophytes

1-3-1- AMPs

When the dermatophyte overrides the innate immune system, dermatophytosis is demonstrated. Antimicrobial peptides (AMPs) are important molecules involved in the innate immunity of the skin having potential antimicrobial effect rapidly killing the microorganisms (28). AMPs are produced by multicellular and unicellular organisms (29-31). Since the skin is the most important organ in contact with the environment as well as the first defense barrier against microorganisms, AMPs are the chemical boundary between host tissue and the environment (32-34). Although the role of AMPs in controlling bacterial growth on the skin surface and protecting the skin against infections caused by bacteriahas been widely recognized, the implication of AMPs in Tinea is poorly understood. It has recently been reported that the level of AMPs is decreased in people afflicted withTinea (35).
Human beta-defensin-3 (HBD-3)and RNase 7 are two essential AMPs inhibiting the growth of dermatophytes in the laboratory (36).
Both AMPs, namely HBD-3 and RNase 7, are stored in lipid within secretory granules called layered bodies in mammals, including humans (37-43). Following skin infection, the layered bodies are opened in keratinocytes, hydrophobic products and AMPs are shed into the middle space and kill microorganisms by decreasing the interstitial fluid (44-46).
Neutrophils and natural killer cells (NKcells) of the skin are rich in AMPs (47-49),which are controlled by the nervous system and secreted as a result of stress and physical injuries (50-52).

2-3-1- Epidermal growth factor receptor(EGFR)

EGFR is a type of membrane tyrosine kinase that plays an essential role in the differentiation, proliferation and invasion of cancers in humans, mostly cancers of epithelial origin (53). In fact, EGFR is like a watchdog in the depth of signals regulating the mentioned factors (54, 55). The expression and activation of EGFR gene are strongly dependent upon several factors preventing the expression and function of EGFR that in fact control it (56-58).

EGFR represents one of the four membranes of HER family of tyrosine kinase receptors, which leads to the binding of binary compound patterns and increases HER family membrane in a variety of cells. In addition, EGFR has several ligands including EGF, TGFA, HB-EGF, EPR, BTC, and AREG (59).
Patients treated with EGFR suppression have been reported to suffer from dermatophytosis (60).
EGFR has been widely described in human cancers including lung (61, 62), CNS (63), head and neck (64), bladder (65), pancreatic (66) and breast (67) and there has been research on diseases that are predicted to have a weaker association with EGFR gene expression (68).
Thus, the inhibition of EGFR may adversely affect the orientation of AMPs in dermatophyte-infected keratinocytes, and blockade of EGFR leads to a dramatic decrease in hBD-3 and RNase 7 in keratinocytes faced with Trichophyton,(Figure 1-2).

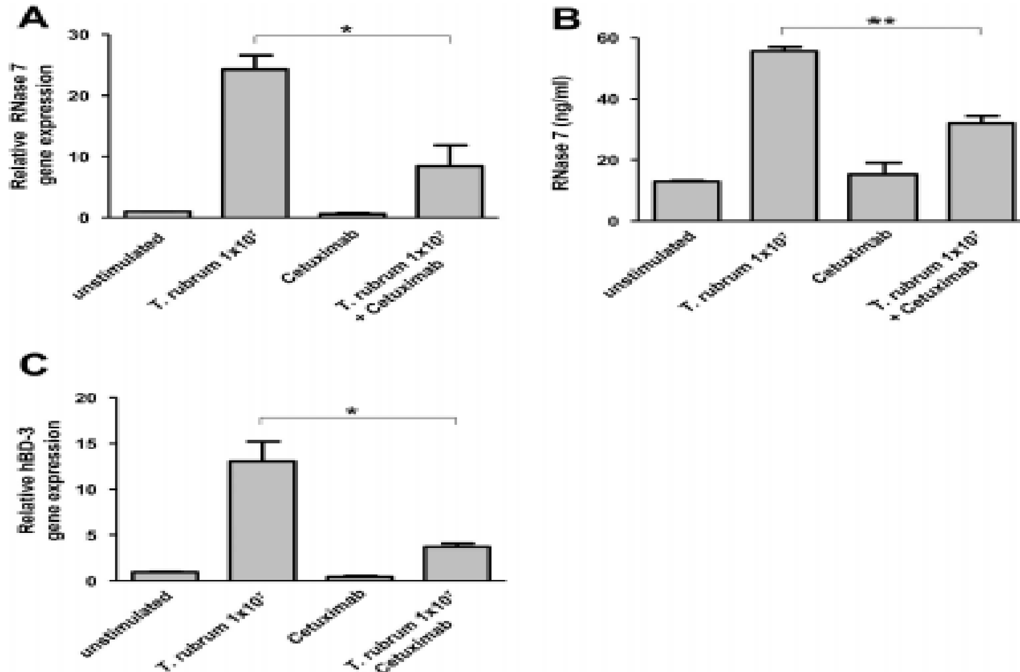


Figure 2-1: The expression of RNase7 and hBD-3 in keratinocytes is dependent on EGFR gene, which is affected by T. rubrum infection (34).

Similar results have been obtained on the anti-EGFR effects in the presence of AMP, which indicate increasing rate of dermatophyte infection in these patients. Higher AMP levels may help the host control the growth and proliferation of other dermatophytes as well as T. rubrum (69).
During fungal infections with T. rubrum, the levels of AMPs such as RNase 7 and hBD-3 are elevated in response to the fungus to prevent growth and inhibit fungal activity as a result of EGFR gene overexpression (70) as shown in(Figure 1-3) and (Figure 1-4).

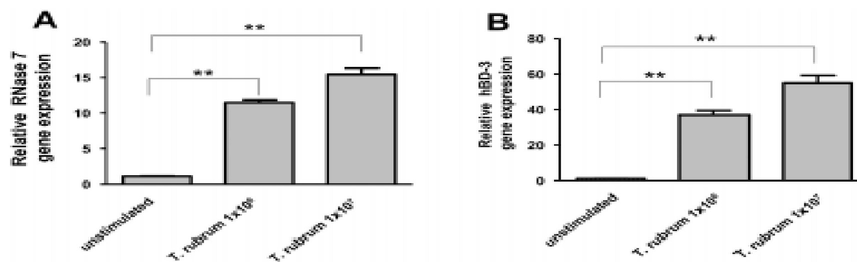


Figure 3-1: Trichophyton rubrum infection is dependent on the expression of AMPs in keratinocytes (34).

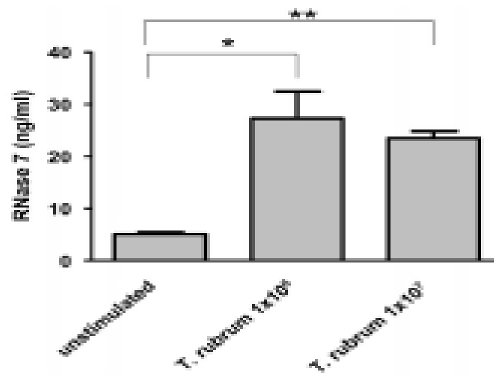


Figure 4-1: RNase 7 secretion in the face of fungus (34).

We decided to argue that the infection with the fungus in question is common, that the diagnosis based on the traditional method of culture on amedium, preparation of culture slides and observation under a microscope is lengthy and takes nearly14-21 days (71).The differentiation of T. rubrum from T. mentagrophytes is difficult due to clinical similarity of the two fungi (72), so we used new molecular methods to detect T. rubrum.
However, because no molecular studies have been conducted with respect to miRNAs in T. rubrum and since the expression of EGFR gene depends on factors such as the presence or absence of microRNAs, we will reveal the role of miRNAs in this study concerning EGFR gene expression to determine whether the dermatophytosis is caused by T. rubrum.

3-3-1- MicroRNAs

1-3-3-1- Structure of microRNAs

MicroRNAs are non-coding RNAs(ncRNAs) with a length of 18-26 nucleotides (73). According to the central dogma of molecular biology, DNA is transcribed into RNA and RNA is then translated into protein. However, this law cannot always express the complexities of an organism because not all DNA sequences encode proteins and the ratio of non-coding sequences to codingones is related to the complexity of an organism, which has an upward trends in higher organisms relative to lower organisms. RNA sequences that are transcribed from DNA but not translated are referred to as non-coding RNAs (74, 75), (Figure 1-5).

Figure 5-1: Law of Central Principle (76)

Recent studies have revealed that more than 90% of the genome is transcribed, while only 1-2% of transcripts are encoded as proteins and 98% of them are called non-encoding RNA. This process, which is present in many species and is associated with complexity of an organism,does not simply waste cell energy but is an important conservative mechanism for cellular function. Thus, contrary to what was previously thought, ncRNAs are transcripts that lack an open reading frame (ORF) and are therefore not capable of producing a protein. These transcripts can be transcribed through one of the three RNA polymerase I, II, and III (77, 78), (Figure 1-6).

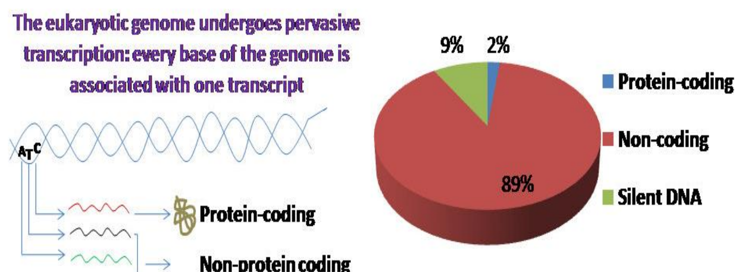


Figure 6-1: Percentage of encoded and non-encoded DNA in the human genome (79)

Nc RNAs are divided into two groups based on size:

1. Small non-coding RNAs
2. Long non-coding RNAs

Based on function, ncRNAs are divided to two groups:

1. Infrastructuralnc non-encoding RNAs, including rRNA, tRNA, small nuclear RNA, and small nucleolar RNA, which have a permanent expression.
2. Regulatory non-encoding RNAs, including microRNAs, small interfering RNAs, long non-coding RNAs, and Piwi-interacting RNAs (piRNAs) (80).

In addition, a new class of ncRNAs, namely promoter-associate RNAs (PARs) and incremental RNAs have recently been added to the group of regulatory ncRNAs (81, 82).

To date, microRNAs arethe most widely studied group of small ncRNAs. These small RNAs are important regulators of post-transcriptional gene expression, play a role in cell regeneration, differentiation, and apoptosis, and are responsible for the maintenance and proper functioning of cells. They have a variety of expression patterns, andchanging expressions of miRNAs has been reported in human cancers, leading to cell transformation and tumor formation (83, 84).

Biochemically, long non-coding RNAs were assumed by Jacob and Monod as mRNAs that do not have the ability to synthesize proteins. Today, the transcripts of long non-coding RNAs have a mysterious but highly important and vital layer in the regulation of protein coding genes. Long ncRNAs are not as conserved as small ncRNAs such as microRNAs or snoRNAs that are highly conserved during evolution (85, 86).

2-3-3-1- Function of MicroRNAs

MiRNAs regulate gene expression by shifting the translation from transcription of the coding protein (73). In this way, these molecules control gene expression after transcription by inhibiting mRNA translation or inducing its degradation by binding to the three prime untranslated region (3'-UTR) at the end of mRNA, and the initial miRNA transcripts are processed in two steps andturn into shorter mature molecules (87-92).
Obviously, as a large subset of non-encoding RNAs,miRNAs are evolutionarily conserved (93).
The important function of miRNA in nematodes was first described in 1993 (94).
The first discovered miRNA was named lineage 4(Lin-4) but the importance of this molecule as a bioregulator was not recognized until 2001 when another example known as Let-7 was identified. In 2001, only five articles were written on miRNAs due to unknown function of miRNAs in small organisms (95), while up to 3,500 papershad been published in Pubmed database by 2008, of which 1,500 articles were related to 2008.Over this period, 6396 miRNA sequences were accepted from the data obtained, but their function was not fully understood (96).
Although the post-transcription function and regulation of mRNAs are known, the mechanism of their post-transcriptional settingis still unknown. Increasing attention is paid to the structure and function of miRNAs due to their effect on various developmental and physiological processes such as apoptosis, insulin secretion, hematopoiesis, brain morphogenesis or tissue differentiation as well as their involvement in immune defense and viral diseases (97-100), suggesting that miRNAs are important biological molecules that have been the subject of much debate (101).
MiRNAs can act as oncogenes or tumor suppressors by inhibiting the expressions of cancer-related target genes (102). A major mechanism of change in all miRNAs of the genome in cancer cells is ectopic gene expression that is detected by abnormal levels of adult microRNAs(92). Therefore, cancer microRNAs can be used as biomarkers for diagnosis, prognosis and even treatment. As mentioned, miRNAs are conserved intracellular ribo-regulator molecules modulating gene expression through the RNA interference (RNAi)pathway. RNAi is a post-transcriptional silencing mechanism in eukaryotes that induces the degradation of homologous mRNAs by generating dsRNA. MiRNAs are partially complemented with the target mRNA and often bind to target 3'UTRmRNA, thereby inhibiting its translation or leading to its degradation. MiRNA genes are dispersed in the genome individually or in clusters, someare found in intergenic regions and at least half of them have been found in specific transcription units such as introns and exons encoding proteins and transcripts that do not encode proteins (90-93, 103-106).

3-3-3-1- Biogenesis of MiRNAs

Biogenesis of MiRNAs is done in nucleus and cytoplasm, (Figure 7-1).

MicroRNA: Mechanism of gene regulation

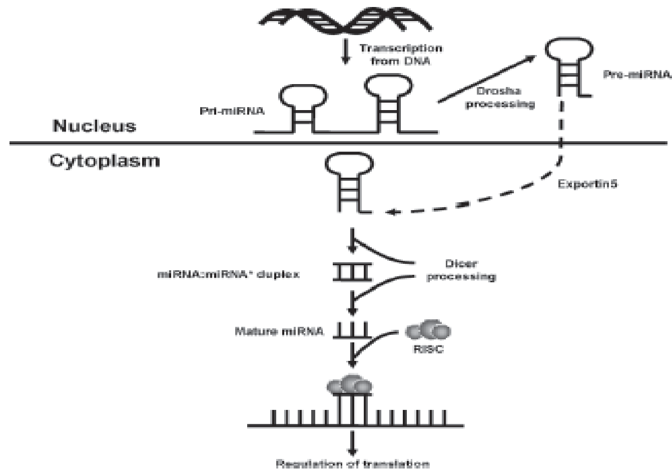


Figure 7-1: Stages of MiRNA evolution (107)

The initial transcript of miRNAs (Pri-mRNA) is several kilobases long, which is transcribed by RNA polymerase II and polyadenylated. This structure is processed by RNase III specific for cleavage of a dsRNA called Drosha and a double-stranded RNA-binding protein known a DGCR/Pasha. DiGeorge Syndrome Critical Region (DGCR) protein is the human protein and Pasha is the non-human one (108). Primary processing leads to the formation of a hairpin loop precursor with 60-110 nucleotides, which is transported to cytoplasm by nuclear exporting factor of Exportin-5 and the auxiliary factor of Ran-GTP. In the cytoplasm, another RNase III called Dicer performs the final processing of miRNA. Dicer cleaves the terminal loop of Pri-miRNA along with a dsRNA binding partner called HIV1-Trans activating response RNA Binding Protein (HIV1-TRBP) in humans, producing the 19-22 nucleotide miRNA (106).
Another TRBP binds the human argonaute protein to the Dicer complex to form an RNA-induced silencing complex (RISC). Only one strand of mature double-stranded miRNA, namely guide, enters into Micro Ribo Nucleo Protein complex (miRNP) and forms a miRNA-induced silencing complex(miRISC), the sequence of which determines the binding site to the target mRNA (108) and the RISC is finally filled with mature miRNAs (109, 110). Proteins of argonaute family are an essential component of RISCs containing two protected domains capable of binding RNA (87). There are pathways in which miRNAs affect gene expression and lead to expression inhibition of their target genes, which is the function of miRNAs on the target gene mRNA. Two important activities of miRNAs have been presented in the following. RISC-binding miRNAs are paired with 3'UTR of mRNA and control gene expression after its transcription or inhibit the translation of target mRNA (A and B in Figure 8-1). Another mechanism is the inhibition of translation by sequestering mRNAs in processing bodies (P-bodies) (part C of Figure 8-1). Thus, mRNAis not translated into protein. This type of mRNA is released in response to natural stimuli, and the translation mechanism is restarted (102, 106), (Figure 8-1).

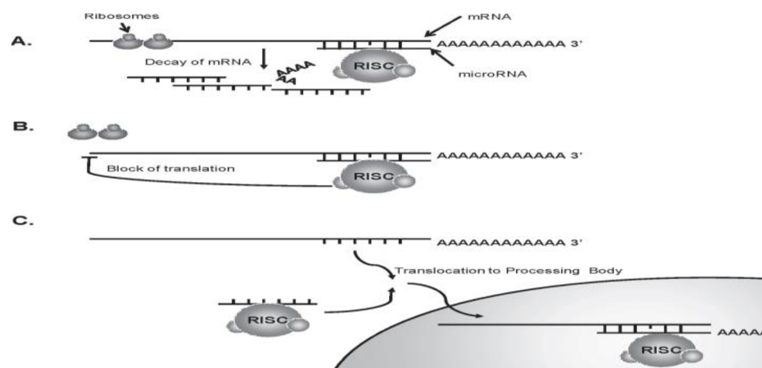


Figure 8-1: Mechanism of translation inhibition by miRNA on target genes via A) Degradation of mRNA B) Inhibition of mRNA translation and C) inhibition of translation by P-bodies(107)

Therefore, the process of translation inhibition by miRNA can be reversible. The complexity of the mentioned processes is a reason for the importance of extensive regulation of miRNAs. One microRNA can target several different mRNAs, or one mRNA may be controlled by several microRNAs; therefore, it is important to identify the target molecules of miRNA to determine the function of miRNA (105).
The way microRNAs affect target mRNAs is shown in (Figure 9-1).

Figure 9-1: Examples of the relationship between different miRNAs and their target tissues (111)

Bernhard's research at the International Cancer Institute in the United States on head and neck squamous cell carcinoma (HNSCC) shows that there is an association between increasing EGFR gene expression and miR-212 gene, so that increased EGFR gene expression is regulated by miRs;in other words, the increase in miR -212 suppresses EGFR mRNAs and eventually reduces EGFR gene expression, a relationship that is true in keratinocytes and is thought to elevate the risk of T. rubrum infection (112).
(Table 5-1) shows the relationship between miR-212 and EGFR genes in head and neck cancer. The regulation of EGFR ligand is specified by a dynamic process, and research into the role of miRNAs indicates that the rapid mechanism of mRNA regulation and gene expression depends on the expression levels of miRNAs and that miR-212 is the most effective among miRNAs.

Table 5-1: MiR-212 among the studied miRNAs has the highest effect on EGFR gene expression

miRNA	Fold Change
has-miR-212	27.8
has-miR-423-5p	9.5
has-miR-483-5p	7.0
has-miR-628-5p	7.0
has-miR-361-5p	5.1
has-miR-95	5
has-miR-342-3p	4.5
has-miR-219-1-3p	4.3
has-miR-491-5p	4.0
has-miR-375	4.0

After extensive research by bioinformatics studies using miRWalk database on the association of EGFR gene with miRNAs(Figure 10-1), we concluded that out of several miRNAs, miR-212 has the greatest effect on the expression of EGFR gene. Therefore, in the following report, miR-212 is nominated for consideration. Given the approach of some of the world's leading pharmaceutical industries to design new drugs based on miRNAs, it is likely that the results of this research will be a small step towards this goal.

miRWalk 2.0: a comprehensive atlas of predicted and validated miRNA-target interactions

EGFR	1956	hsa-miR-21	hsa-mir-21	17	20048743
EGFR	1956	hsa-miR-21	hsa-mir-21	17	19493678
EGFR	1956	hsa-miR-21*	hsa-mir-21	17	22213426
EGFR	1956	hsa-miR-21*	hsa-mir-21	17	19597153
EGFR	1956	hsa-miR-21*	hsa-mir-21	17	21063030
EGFR	1956	hsa-miR-21*	hsa-mir-21	17	20113523
EGFR	1956	hsa-miR-21*	hsa-mir-21	17	22766763
EGFR	1956	hsa-miR-21*	hsa-mir-21	17	20048743
EGFR	1956	hsa-miR-21*	hsa-mir-21	17	19493678
EGFR	1956	hsa-miR-210	hsa-mir-210	11	19493678
EGFR	1956	hsa-miR-212	hsa-mir-212	17	20856931
EGFR	1956	hsa-miR-216a	hsa-mir-216a	2	22213426
EGFR	1956	hsa-miR-216b	hsa-mir-216b	2	22213426
EGFR	1956	hsa-miR-217	hsa-mir-217	2	22213426
EGFR	1956	hsa-miR-221	hsa-mir-221	X	22213426
EGFR	1956	hsa-miR-221*	hsa-mir-221	X	22213426
EGFR	1956	hsa-miR-25	hsa-mir-25	7	20818338
EGFR	1956	hsa-miR-25*	hsa-mir-25	7	20818338
EGFR	1956	hsa-miR-27a	hsa-mir-27a	19	22213426
EGFR	1956	hsa-miR-27a	hsa-mir-27a	19	21156786
EGFR	1956	hsa-miR-27a*	hsa-mir-27a	19	22213426
EGFR	1956	hsa-miR-27a*	hsa-mir-27a	19	21156786
EGFR	1956	hsa-miR-27b	hsa-mir-27b	9	22213426
EGFR	1956	hsa-miR-27b	hsa-mir-27b	9	22902387
EGFR	1956	hsa-miR-27b*	hsa-mir-27b	9	22902387
EGFR	1956	hsa-miR-27b*	hsa-mir-27b	9	22213426
EGFR	1956	hsa-miR-296-3p	hsa-mir-296	20	22075979
EGFR	1956	hsa-miR-296-5p	hsa-mir-296	20	22075979
EGFR	1956	hsa-miR-30a	hsa-mir-30a	6	18668040
EGFR	1956	hsa-miR-30a*	hsa-mir-30a	6	18668040
EGFR	1956	hsa-miR-30b	hsa-mir-30b	8	18668040
EGFR	1956	hsa-miR-30b*	hsa-mir-30b	8	18668040
EGFR	1956	hsa-miR-30c	hsa-mir-30c-1	1	18668040

Figure 10-1: Data retrieval from miRWalk database on microRNAs targeting miRNAs

4-1- Real-Time PCR technique

In general, Real-Time PCR is a technique for continuously observing the progress of PCR reaction over time. Indeed, it is a combination of measuring the fluorescent light with PCR. In this way, the amount of PCR product is measured using quantitation of fluorescent light. Amplification of the target fragment can be detected by the device when the intensity of light emitted from the product in the initial phase is higher than that emitted from the background. In other words, the fluorescencelevel increases until it reaches a threshold that is higher than the background to a certain degree. This stage of the reaction is known as cycle threshold(Ct, the cycle in which Real-Time PCR product surpasses a threshold) that is referred to as crossing point, namely the beginning of copying the template that is used in the calculation of test results.Ct has a significant relationship with the value of the original template, which can be used to estimate the primary mRNA level. The higher the gene expression, the fewer cyclesneeded to produce more product and thus the lower the Ct. This method is based on fluorescence or in the form of dyes binding to double-stranded DNA(such as SYBR Green) or in the form of specific sequence detectors such as TaqMan probes. In fact, by producing a double-stranded PCR product, the dye molecules are inserted between the ds-DNA bases and begin to emit. Therefore, the higher the concentration of the product, the higher the intensity of radiation. This technique can start with a minimum amount of nucleic acid and determine the level of final product with great accuracy. The technique of this device is derived from conventional PCR, with the difference that the method of detecting and analyzing the results is different and much more accurate than the conventional PCR method with electrophoresis. Real-Time PCR has the ability to detect PCR expansion in the initial stage of the reaction, while the detection in conventional PCR is done in the final

stage of reaction. In this method, there is no need to use gel to observe bands and the results are immediately displayed at the end of each cycle in two sigmoidal and logarithmic diagrams (Figure 11-1).

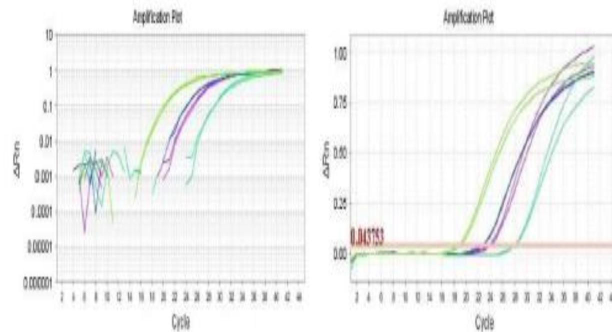


Figure 11-1: Sigmoid (right) and logarithmic (left)plots drawn by Real-Time device. Moreover, quantitative measurement using this technique has high precision and is thus called qPCR relative to the conventional semi-quantitative PCR that has low precision

SYBR Green is the most common dye used in this technique. It is an intercalating dye anda fluorescent reporterconnecting to small grooves of ds-DNA, emitting a 522 nm wavelength by absorbing 498 nm that is recorded by the device. SYBR Green does not bind single-stranded templates and has thus low emission in this regard. The dye binds the double-stranded PCR product produced in PCR cycles and therefore the increase in fluorescence intensity is proportional to dsDNA concentration. SYBR Green is a nonspecific dye that can be used for all tests,which is considered an advantage. Other advantages include inexpensiveness, non-interference with polymerases, and non-toxicity. SYBR Green remains stable under PCR conditions. As mentioned, SYBR Green is non-specific,an advantage that is also its most important disadvantage because the presence of non-specific products or dimer primers cannot be detected using this dye. Obviously, the use of melting curve diagrams in today's devices has largely solved this problem. The melting curve should be used to find out whether PCR functioned properly. For this purpose, a temperature below the annealing temperature up to a temperature >95°Cis considered and the relevant program set for the device, so that the lamp is turned on for every 0.5°C increase in temperature to read the intensity of fluorescent absorption,which leads to the absorption peak to be plotted for all amplified fragments. In this way, the contamination is detected by observing the relevant absorption peak. At low temperatures, the plotted peaks related to the starting dimer and the last peak are related to our desired fragment. A favorable temperature leads to the induction and emission of wavelengths.

In general, Real-Time PCR has several stages, including baseline region or linear phase, in which there is no light emission despite the presence often double-stranded product;exponential phase and log linear phase, in which the double-stranded product doubles in each cycle and the reaction-related exponential growth begins;plateau phase where the reaction compounds are destroyed and no increase in fluorescence is observed.(Figure 12-1) shows the amount of product amplified in Real-Time PCR in terms of PCR cycles.

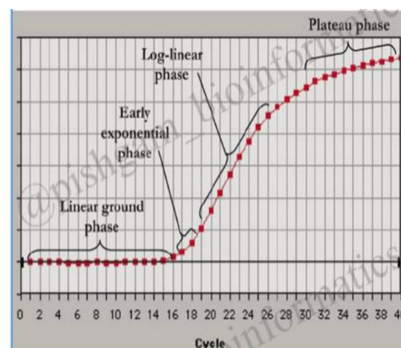


Figure12-1: Real-Time PCR steps

The diagrams are plotted by the device in terms of change in fluorescence (ΔRn) per number of cycles (C) and displayed on computer monitor screen connected to the device. The change in fluorescence is the main parameter by which the device draws diagrams. In this method, instead of final staining of PCR products in each cycle and viewing them with UV, the fluorescence is increased proportionate to the increase in products, which is

recorded by the device camera at the end of each cycle and displayed on the diagram. Finally, the completed diagrams can be analyzed (113-115).
In Figure 13-1, Ct shows a point on the diagram where it meets the baseline. Threshold is a line drawn horizontally by the device and it is usually placed at the beginning of the incremental phase of PCR. By comparing the Cts between test and control samples, the difference between the two samples can be determined with high accuracy, (Figure 13-1).

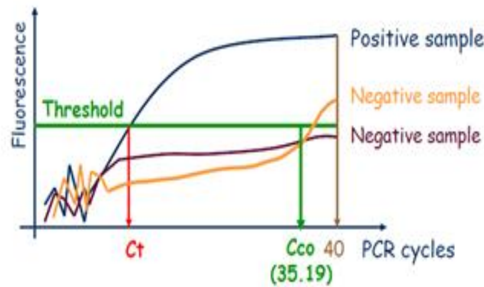


Figure 13-1: Ct specification methods

Real-Time PCR reaction can be single- or two-step.In single-step Real-Time PCR, cDNA synthesis and amplification is achieved in one tube. In the two-step reaction, reverse transcription and amplification take place in separate tubes. In a single-step Real-Time PCR, laboratory changes are minimized but it is less sensitive. In the two-step method, different parts of a target DNA can be measured and it is easier to remove the dimer primers by manipulating the annealing temperature. Although it may increase the chance of contamination, the two-step method has more advantages than the single-step one, (Figure 14-1).

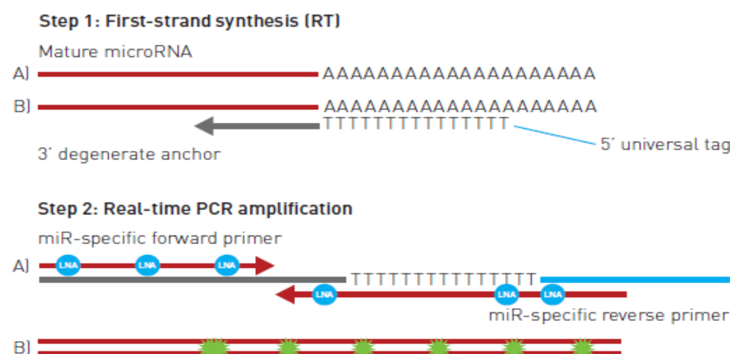


Figure 14-1: Two-step Real-Time PCR(113)

5-1- Objectives of the project

1- 5-1- The main goal of the project

Determination of changes in miR-212 and EGFR genes expressions in clinical samples infected with T. rubrum dermatophytes in involvedsites compared to adjacent healthy ones.

2-5-1-Partial objectives of the project

1. Determining EGFR gene expression in cells infected with T. rubrum
2. DeterminingmiR-212 gene expression in cells infected with T. rubrum
3. Determining the relationship between EGFR and miR-212 genes expressions in cells infected with T. rubrum
4. Assessment of EGFR and miR-212 genes expressions in healthy cells present at the margin of infection that have not been infected

3-5-1- Practical goals of the project

The results of this research can be helpful in designing diagnostic and therapeutic tests based on the mentioned genes or in controlling the disease.If researchers are to design diagnostic and therapeutic kits based on miRNAs, this investigation can be a small step in line with this big goal and such investigations are needed to design these kits.

6-1- Research hypotheses or questions (according to objectives of the project)

1.Is there a relationship between the expression of EGFR gene with T. rubrum infected cells?

2. Is there a relationship between the expression of miR-212 gene and T. rubrum infected cells?
3. Is there a relationship between the presence and increasing expression of EGFR gene with immunity of the cellto the infection with the fungus?
4.Is there a relationship between miR-212 activity and the resulting suppression of EGFR gene?

7-1-Summary of research method

1. Collection of clinical samples after the consent of patients referred to Razi Dermatology Hospital affiliated to Tehran University of Medical Sciences, and basically 36 samples of tissue infected with T. rubrum were collected.
2. Collection of 36 seemingly healthy control samples from the margin of the lesion related to the fungus-infected parts in the same patients.
3.Extraction and optimization of total RNA
4. Synthesis of cDNA and its optimization
5. Amplification and expression of EGFR and miR-212 genes by Real-Time PCR (SYBR Green)
6. Data collection and analysis
7. Comparison of results with clinical pathological findings

CHAPTER II

REVIEW OF LITERATURE

According to the Introduction, extensive research has been conducted on the presence and involvement of miRNAs in gene expression, especially in the field of cancer. The following are just a few examples of research efforts that have taken steps in the field of modern science to identify miRNAs and their function.

- Hiromitsu Hatakeyama et al. in 2010 studied the regulation and expression of EGFR gene by inducing the expression of miR-212 in HNSCC and concluded that miR-212 silences the EGFR gene, which results in HNSCC (112).

- In 2014, Helene Firat Yasemin and colleagues examined AMPs (HBD-3 and RNase7) that are dependent on the expression of EGFR gene and provided a detailed report on EGFR gene expression in the skin leading to the appearance of these AMPs. The AMPs in keratinocytes resist the attack by T. rubrum and prevent dermatophytosis with this fungus or even other dermatophytes (34).

- Soheila Rahgozar et al. in 2011 presented a report on miRNAs with altered expression levels that are involved in different human cancers (116). They examined several miRNAs, some of which we will mention here. miR-194, miR-192, miR-200c, and miR-21 showed increasing expression but the expression of miR-203 and miR-205 was decreased. In endometrial adenocarcinoma, the expression of miR-205, miR-449, miR-429 increased and that of miR-193a, miR-204, and miR-99b decreased. In their research, the identification of miRNAs and their target molecules has been introduced as a promising approach for identifying the pathways leading to cancer. The researcher cites miRNAs as potential biomarkers in the diagnosis, prognosis and treatment of cancer (117).

- A study by Shahrokh Irvani in 2012 examined the association between gastric cancer and Helicobacter pylori infection. The researcher explained this relationship based on host and environmental factors such as nutrition and health status. With respect to the genetic background of gastric cancer, the researcher argued that the genetic background, which refers to the set of characteristics of an individual at the genome level, is a factor causing cancer (118). The main components of the genetic background are mutations and polymorphisms that exert their effect by altering the expression or function of proteins (119). Cancer is caused by alterations in oncogenes, tumor suppressors and microRNAs. In other words, the researcher assumes that cancer presentswith high expression of specific miRNAs, attributing the inflammatory process of bacterial infection as well as inflammatory response of the body to genetic susceptibility of the individual for developing cancer in gastric cells (120).

- A study conducted by Mohammad Amin Honardoust et al. in 2013 on the expression rate of miR-26a in peripheral blood mononuclear cells of patients from Isfahan with relapsing-remitting MS (RRMS) during recurrence and improvement stages compared to healthy individuals showed that Th17 cell subtype and thereby the expression level of IL-17 gene in PBMC increases in the recurrence phase compared to the recovery phase. The researcher first performed bioinformatics studies predicting mRNA-miRNA interaction in miRNAs with altered expression in autoimmune disease as well as positive and negative regulators of miR-26a, which introduced Th17 as the miRNA that can be an inducer for differentiation of this cell line probably by inhibiting the translation of negative regulators of Th17 cell subclasses such as phosphatase and tensin homolog (PTEN) and Tuberous sclerosis 1 (TSC1). The results showed that the relative expression of miR-26a in the recurrence phase of RRMS increased significantly compared to the recovery phase and also in comparison with healthy individuals, which confirms the induction role of miR-26a in the differentiation of Th17 cell line. Th17 is a subset of $TCD4^{+}$cells that has a pathogenic role in the development of autoimmune disease (121).

- In another study by Mohammad Amin Honardoost and colleagues in 2013, patients with RR-MS were examinedwith sudden onset and exacerbation of symptoms (relapse phase) following partial or complete recovery (remission phase) (122-124). This time, however, they examined miR-155 and miR-326 (125) and stated that most miRNAs subject to altered expression in the blood or cerebral plaques of MS patients are regulators of genes involved in physiological and pathological processes of cells residing in the brain and thatmiR-155 is essential for the production of gammaglobulin (IgG) (126-128). Furthermore, following the activation of $TCD4^{+}$ cells, miR-155 expression is significantly increased, indicating the importance of miR-155 function in active$TCD4^{+}$cells (125). Transcription factors such as C-MaF and suppressor of cytokine signaling 1 (SOCS1) as well as cytokines and signaling proteins are among the target proteins of miR-155 (130). MiR-326 is highly upregulated in active MS plaques and blood of people with MS compared to healthy individuals (131, 132). MiR-326 seems to play an essential role in the pathogenesis of MS by inducing the differentiation of Th17 cells both in vivo and in vitro. MiR-326 has been shown to target the ETS proto-oncogene 1, transcription factor(ETS-1) (132).

- Mina Noormohammadi and colleagues in 2014 also conducted a research on changes in the expression of miR-222 in gastric cancer associated with H. pylori. Based on bioinformatics data, miR-222 was found to be associated with proliferative processes, programmed cell death, differentiation, and migration due to strong bond formation and MAPK signaling pathway. Disruption of various biological processes can lead to gastric cancer. The results of their study showed that the expression of miR-222 is increased in gastric cancer with H. pylori infection and suggested that miR-222 is an OncomiR and may serve as a biomarker of cancer development (133).

- Hajar Miranzadeh Mahabadi et al. in 2013 presented a report on the common metabolic syndrome in children, which is associated with a set of risk factors such as obesity, diabetes, increasing triglyceride levels, high blood pressure and low HDL cholesterol. They stated that miR-33b gene, along with its host gene of Sterol Regulatory

Element Binodig transcription Factor 1 (SREBF-1), is a major regulator in maintaining the unstable cholesterol state of the cell by regulating the insulin signaling pathway. Therefore, miR-33b can be considered as a candidate gene for metabolic syndrome. Using real-time PCR technique, the researcher proved that there was no significant relationship between miR-33b gene with the risk of metabolic syndrome in children and adolescents of Isfahan (134).

- A 2016 study by Barker et al. showed that miR-155 is fully involved in the host's immune response to infection and neuroinflammation through negative regulation in blood brain barrier (BBB) and T-cell function. They believed that miR-155 may be involved in the pathogenesis of cerebral malaria (CM) and expressed miR-155 gene experimentally in a model of laboratory cerebral malaria (ECM) using the serum samples of Ugandan children with malariatogenerate anti-miR-155 antibody as a potential treatment.The researchers attributed the survival of mouseto maintaining BBB integrity at a higher parasitological leveland endothelial activity decreased despite increasing levels of inflammatory cytokines. Before treatment, miR-155 leakage from veins is decreased, leading to cerebral malaria in humans.These findings provide evidence to support the role of miR-155 in the host's response to malaria and endothelial activity (135).

- Schistosoma japonicum is one of the most serious zoonotic diseases in the world.Therefore, in a study conducted by Hong et al. in 2017 on changingmiRNA expression in response to infection with Schistosoma japonicum, it was stated that there is evidence on increased miRNA expression in models infected with the parasite. Several miRNAs have been proposed for potential diagnosis or for new therapeutic purposes for Schistosoma japonicum, developing newer approaches to control this disease (136).

- In a 2017 study by Young et al. on the role of miRNAs inHBV, it was shown that HBV(a DNA virus) encodes miRNAs by an unknown mechanism. This microRNA, which was named HBV-miR-3, is located at 373-393NTS locus of HBV genome. In patients with HBV infection, there is an increase in the expression of HBV-miR-3 in hepatoma cells, which manifests itself in the form of hepatoma (137).

- In a study conducted by Casslli T. et al. in 2017, the role of miRNAs and their relationship with mRNA in Borrelia burgdorferi (Bb) infection was discussed. Infection with this bacterium manifests in the form of rash or systemic symptoms. However, the bacterium can invade other tissues and cause joint pain or neurological symptoms similar to Bell's palsy. The researcher observed significant changes in 38 miRNAs as well as in 275 mRNAs within 24 and 48 hours after experimental Borrelia infection. The changes in several RNAsinvolved in the immune response affects cell development and adhesion. Finally, it was shown that the exposure to Bb leads to significant changes in the transcript and profile of astrocyte miRNAs, which has implications for pathogenesis and potential treatment strategies for the disease (138).

- Lup et al. in 2017introduced a Treponema pallidum, namely the bacterium that cannot be cultured and causes acute and chronic syphilis, which was not capable of producing toxic proteins. Therefore, many symptoms of disease and tissue damage wereattributed to the activation of host immune responses,indicating that host’s genetic background plays an important role in regulating syphilis infection.The researchers discussed the role of miRNAs in their report (139).

- In a 2016 study by Hejj et al. the expression of miRNA genes in the spleen of mice exposed to Toxoplasmagondii was examined and it was found that 131 out of 379 isolated miRNAs had different expressions, including 97 with positive and 34 with negative regulation. By analyzing the gene, the researchers stated that Toxoplasma can regulate the expression of the host gene by targeting transcriptional regulatory factors. The genes involved in apoptosis or anti-apoptosis are both targets of different miRNAs expressed in this disease. Changing balance of miRNAs, targeting the host’s apoptosis gene and those hosting the regulation of anti-apoptotic gene determine the patient's fate in terms of infection with Toxoplasma gondii. The researchers reported a link between infection with Toxoplasma gondii with gene regulation, apoptosis, and changes in the malignant process in the spleen of infected mice (140).

Inspired by the mentioned studies in disciplines other than mycology, we tried to contribute to the relatively overlooked field of mycology by taking steps in finding novel methods of disease discovery in mycology. We hope to be able to rapidly diagnose dermatophytosis due to T. rubrum given the high incidence of this fungus and help treat it.

Chapter III

MATERIALS AND METHODS

Table 1-3: Equipment used for sampling and performing molecular tests related to Real-Time PCR

Company	Country	Equipment
Domestic companies	Iran	Ethanol
Domestic companies	Iran	Cotton
Domestic companies	Iran	Sterile disposable plates
Domestic companies	Iran	Sterile glass plate containing U-shaped tube for slide culture
Domestic companies	Iran	Disposable glove
Domestic companies	Iran	Powder-free sterile glove
Domestic companies	Iran	Microscope slide and coverslip
Domestic companies	Iran	Blue sampler head
Domestic companies	Iran	Yellow sampler head
Domestic companies	Iran	Crystal white sampler head
Domestic companies	Iran	Blue sampler head rack
Domestic companies	Iran	Yellow sampler head rack
Domestic companies	Iran	White crystal sampler head rack
Domestic companies	Iran	2 mL microtube rack
Griener	Germany	2 mL cryotube
Griener	Germany	2 mL microtube
Griener	Germany	1.5 mL microtube
Griener	Germany	DNase and RNase free2 mL microtube
Roche	Germany	Light cycler 8 Tube strips with PC 2mL microtube

Table 2-3: Devices used to perform mycological and molecular tests related to Real-Time PCR

Company	Country	Device
Ront gen	Germany	-70°C Freezer
Domestic companies	Iran	-20°C Freezer
Domestic companies	Iran	Electrophoresis tank
Nicon	Japan	Microscope
E-Box	Germany	UV-doc system
IKA-WERK	Germany	Mixer or vortex
Domestic companies	Iran	TECHNOFER bain-marie
Accu LAB	America	Scales
Eppendorf	Germany	Sampler
Eppendorf	Germany	Refrigerated centrifuge
Eppendorf	Germany	Refrigerated micro-centrifuge
Eppendorf	Germany	Thermocycler
Thermo	Germany	Nanodrop
Roche	Germany	Real Time

Table 3-3: Materials used to identify and differentiate T. rubrum and Real-Time PCR molecular test kits

Manufacturer or Importer	Country	Material
Merck	Germany	Sabouraud dextrose agar medium
Merck	Germany	Urea agar medium
Merck	Germany	Cornmealagar medium
Sinaclon	Iran	RNA isolation Kit
Sinaclon	Iran	RNAse-free DNAse 1 kit
Aminsan –Takara	Japan	cDNA synthesis kit
SakanMed –Exiqon	Denmark	MicroRNA amplification kit
VIRAGENE –Ampliqon	Denmark	Master mix PCR kit
Sinaclon	Iran	EGFR gene primer
Sinaclon	Iran	GAPDH gene primer
Exiqon	Denmark	MiR-212-3p gene primer
Exiqon	Denmark	MiR-103a-3p gene primer
Sinaclon	Iran	Nuclease-free water
Thermo	Germany	EDTA
Thermo	Germany	Agarose powder
Thermo	Germany	TBE-1X buffer
Thermo	Germany	Looding Dye

Method

1-3- The process of fungus identification with the help of mycological tests

1-1-3- Sample collection

The collection of skin lesions in this project was done in collaboration with Razi Dermatology Hospital affiliated with TUMS. Due to the fact that our research was concerned with skin samples, we focused only on skin samples and omitted nail and hair samples.

First, the sample scratch and control site were disinfected with 70% alcohol. Samples were taken from both patient and control. To prepare patient sample, a scalpel was used to cut fungus exactly from Tinea lesion where it was active and had satellite growth, which was poured into a cryotube. If the-70°C freezer was available, the sample was transferred to the freezer immediately without the slightest delay because our review was conducted on RNA that can be easily degraded due to the presence of RNAse in the environment. If the-70°C freezer was not available at the sampling site, the samples were poured into a cryotube and transferred to -196°C liquid nitrogen.Fortunately, there was no need for liquid nitrogen because -70°C freezer was available in mycology sampling section of Razi Hospital. The control sample was taken from the same patient from whom the lesion sample was removed.At a distance of 15-20 cm from the lesion, the intact skin of the participant was shaved, poured into the cryotube and immediately transferred to -70°C freezer.It is worth mentioning that the consent form was submitted to all the subjects and all patient data were archived in full confidentiality with the ethical code received from Kerman University of Medical Sciences.Simultaneous with taking a sample from the lesion, some of the shaved skin was transferred to a plate with Sabouraud dextrose agar containing cyclohexamide (Aktdyn) and chloramphenicol to identify the fungus after its growth.

2-1-3-Preparation of Sabouraud dextrose agar medium

In Sabouraud dextrose agar medium with pH=6.8-7, the dextrose content is 20%. To inhibit the growth of saprophytic fungi, 500 mg of cyclohexamide was dissolved in 10 mL of acetoneand added to 1 L of culture medium. In addition, to prevent the growth of bacteria, 50 mg of chloramphenicol antibiotic was dissolved in 10 mL of alcohol and added to 1 L ofthe above mentioned medium. A medium called Mycosel agar or Mycobiotic is commercially available. 65 grams of the medium powder was weighted and added to1 liter of distilled water, then the medium was boiled while shaken with magnet until transparency,divided into tubes and placed in an autoclave to sterilize. Alternatively, after sterilizing the medium, it was divided into sterile disposable plates. The fungus needs a temperature of 25-30°C to grow and it will not grow at 37°C (141). Phosphorus and nitrogen containing compounds inhibit the growth of this fungus (142).

3-1-3- Differentiation between T. rubrum and T. mentagrophytes

It should be noted that the following methods are used to distinguish between T. rubrum and T. mentagrophytes due to the high similarity of clinical symptoms as well as colonies of the two fungi:

1. Christensen urea agar contains peptone, sodium chloride, dipotassium phosphate, glucose, agar, an indicator (phenol red) and distilled water. Phenol red turns yellow (colorless) in acidic medium and pink (purple) in alkaline medium. T. rubrum does not change the color of the medium because it is urease negative.
2. Another way to differentiate between T. rubrum and T. mentagrophytes is to use Cornmeal Agar medium, which stimulates the production of a purple-red pigment by T. rubrum, while T. mentagrophytes does not produce any medium after growing on this medium.
3. The third method to distinguish these two fungi from each other is the hair perforation test, in which T. mentagrophytes perforates autoclaved hair in vitro but T. rubrum does not.

4-1-3- Observation of microscopic structure of fungus

Slide culture method was used to prepare a slide from the colony of fungus in the following steps:

1. A small portion of Sabouraud dextrose agar was placed on a slide lying on a U-shaped glass tube inside the plate. This process should be done next to the flame and in a completely sterile manner.
2. The colony of fungus was cultured on four parts (sides) of agar using a sterile swab in the form of slide culture.
3. The inoculated square surface was covered with a sterile coverslip.
4. 8 mL of sterile distilled water was added to the bottom of the plate to sterilize the air inside the plate.
5. The slide culture was placed at an appropriate temperature (25-30°C) and checked every few days for the required features without disturbing the growth of fungus.
6. The square-shaped agar was removed from the slide, both the slide and coverslip were dipped with one drop of lactophenol cotton blue (LCB), a coverslip was placed on the slide (or the slide was placed on coverslip) and gently heated to remove the air bubble (141).
7. Then, under a microscope, the hyphae, microconidia and macroconidia of the fungus were observed and judged whether the fungus was T. rubrum based on the appearance of the fungus,which was discussed in detail in Introduction.

After ensuring that the fungus wasT. rubrum, it was time to use the flakes in the cryotube within -70°C freezer to perform molecular tests.

2-3-The steps of molecular tests and the introduction of kits

- At the beginning of the work to separate RNA and all molecular tests, the desktop and all samplers were disinfected and cleaned with 70% alcohol.
- All work equipment, including sampler heads, racks, and microtubes were autoclaved.
- Disposable gloves and powder-free sterile latex gloves were used.
- It is better to use a mask because RNase enzyme is abundant in the environment and may affect the final result.
- As shown in the list of devices, a refrigerated centrifuge was used with temperature set at 4°C in all stages of work and the temperature was kept stable.
- The use of ice in the work process is recommended.

1-2-3 -Total RNA extraction

1-1-2-3- The contents of RNX-Plus kit from Cinnagen (Made in Iran) for total RNA isolation

1. Assembled mini spin columns
2. Collection tubes
3. Lysis Buffer
4. Precipitation Buffer
5. Wash Buffer I
6. Wash Buffer II
7. RNase free water

2-1-2-3- Kit precautions

1. The solutions in the kit should not have contact with the skin or eyes.
2. Gloves should be worn when doing work because skin is an important source of RNase.
3. Lysis buffer and rinsing buffer I are irritating.
4. Contact of lysis solution with acid or bleaching solutions produces toxic gas.

3-1-2-3- Kit keeping conditions

The kit is stable at 18-25°C, but the company's recommendation is to store it at 2-8°C in refrigerator.

4-1-2-3- Introducing the kit

RNX-Plus kit system is one of the latest nucleic acid purification technologies. The basis of this technology is the binding of RNA to materials containing silica membrane filters with a high salt concentration and can be reversibly washed at low salt concentrations such as RNase free water.

5-1-2-3- Steps of the research

1.25-50 mg of the sample should be removed. Since our samples were shaved skins, this step was not necessary (10 mg of sample with active RNA is sufficient).
2.The sample should be pulverized.Because our samples were small enough while shaving the patient's skin, there was no need to pulverize the sample.
3.The sample was poured into a 2 mL sterile microtube.
4. 400 λ of lysis solution was added to the sample.
5. Using a vortex or mixer, the sample was mixed for one minute.
6.The sample was homogenized by ten times shaking in a syringe
7. 300 λ of precipitation solution was added, the lid closedand shaken again.
8.The contents of the microtube were transferred onto the column.
9.The column was centrifuged at 1300 RPM for 1 minute.
10.The contents under the column were discarded.
11. 400 λ of rinsing solution I was poured into the column, centrifuged at 1300 RPM for 1 minute, and the solution under the column was discarded again.
12. 400 λ of rinsing solution II was poured into the column, centrifuged for 1 minute at 1300 RPM and the solution under the column was discarded.
13. Step 12 was repeated.
14.The column with its tube was centrifuged at 1300 RPM for 2 minutes.
15. The column was transferred into a 2 mL sterile microtube.

16. 50 λof RNase free water (55°C) was added, the lid closed and incubated at 55°C in thermocyclerand centrifuged at 1300 RPM for one minute.
17. The column was discarded and what remained in the microtube after centrifuge was total RNA.
18. The RNA-containing microtube was kept on ice until it was placed in-70°C freezer and the concentration of RNA was read using Nanodrop device (Figure 1-3).

Figure 1-3: Nanodrop device

Determining the concentration with Nanodrop device is a quantitative method and the concentration and purity of RNA sample can be determined using light absorption at 260 and 280 nm.
Nucleic acids are absorbed at 260 nm. The protein is absorbed at 280 nm and other contaminants such as phenol, chloroform, and the like are absorbed at 230 nm.
In this method, first the blank is setusing RNAase free water, 2 μL of RNA is loaded directly on the device probe and the concentration of the sample is calculated by determining the absorbance at 260 nm.
A260/A280 ratio is used to measure the purity of RNA. The proper ratio for pure RNA is 1.2-1.8 and higher ratios indicate contamination with protein or phenol. The results obtained from this step indicate that the extracted RNA can be used with high confidence in later stages of the research.
19. After reading the absorbance, total RNA was immediately transferred to -70°C freezer.

6-1-2-3- Kit instructions

1. Avoid contamination of the columns with alcohol.
2. Make sure the columns are dry.
3. The final volume after washing depends on the amount of the original sample:
   A) If the amount of RNA is high, the final volume will increase to 200 uL.
   B) Concentrated RNA is obtained when the final volume after washing is <30-30 uL.
4. In a standard measurement, the absorption ratio (Q) should be read between 260 and 280 nm. However, at lower concentrations of RNA,the absorption may read between 170 and 200 nm.
5. The coefficient obtained with 260-280 nm absorption should be ≥1.9.
6. In cases where DNA is likely to remain in the final volume, the DNase treatment method should be employed.

2-2-3- Elimination of genomic DNA contamination from purified RNA

DNase I is an endonuclease that can digest single- and double-stranded DNA. This enzyme hydrolyzes phosphodiesterase bonds to form mononucleotides and oligonucleotides with 5´-phosphate and 3´-OH groups. The function of this enzyme is dependent upon Ca^{2+} ions and it is activated in the presence of Mg^{2+} and Mn^{2+} ions. DNase I treatment is necessary before RT-PCR and qPCR reactions.

DNase Treatment: RNA treatment with DNase I enzyme

In general, to remove the part of genomic DNA that is not removed during RNA extraction and is associated with it, DNase I enzyme and DNase treatment process are needed to avoid further problems by eliminating this genomic DNA. By designing PCR primers so that they are placed on two different exons, the length of the amplified DNA fragment will be longer and it will be more recognizable because of containing introns,and this step will augment precision. In this study,DNase I, RNase free kitof Cinnagen Company (made in Iran) was used.

1-2-2-3- Materials

Table 4-3: Preparation of materials for RNase free, DNase I reaction

RNA	1 µg
10X Reaction Buffer with MgCl2	1µL
DNase I, RNase free	0.5 µL
DEPC-treated Water[1]	to 10µL
EDTA 50 mM	1 µL

2-2-2-3- Steps of the research

1. An appropriate amount of extracted RNA (based on the concentration and purity of extracted RNA by Nanodrop) was added to 0.5-mL microtubes free of RNase enzyme.

2. 0.5 µL of DNase I enzyme and 1µL of its 10x buffer was added to each microtube and the final volume reached 10 µL.

3. The tubes were incubated at 37°C for 30 minutes in the thermocycler.

4. 1 µL of 50 mM EDTA solution was added and incubated at 65°C for 10 minutes or at 80°C for 2 minutes in the thermocycler.

3-2-3- Preparation of agarose gel (qualitative examination of the extracted RNA)

Before electrophoresis of RNA sample, all electrophoresis equipment were kept for 15 minutes in 0.5 M NaOH solution and after complete washing with water, they were incubated with H_2O_2 solution (3%) for 15 minutes. Finally, all equipment were rinsed twice with double distilled water.

Then, the appropriate amount of agarose powder was weighted and dissolved in the desired volume of TBE-1x buffer. To prepare 1% agarose gel, 1 gram of agarose was weighted and dissolved in 1 ml of 1x buffer. The solution was heated until it boiled and the agarose was completely dissolved in it. After cooling, the gel was gently poured into the electrophoresis container in which the combs had already been placed. After firmness of the gel, the combs were removed and the tank filled with TBE-1x. Afterward, the desired amount of RNA was mixed with loading dye and inserted into gel wells. The voltage (70 volts for RNA) was set after pouring all the sample stogether with the molecular weight marker. After this time, the gel was washed and observed in UV-Doc device. The RNA sample, which is chemically intact with standard biological quality, shows a special bonding pattern on the agarose gel.The presence of S18 and S28 ribosomal RNA bands indicates that the RNA is intact. High-quality samples show the minimum smear above, between, and below the mentioned bands, and the intensity of S28 bands is almost twice that of S18 band. The absence of clear S18 and S28 bands indicates that the RNA sample

1 Di Ethyl Pyro Carbonat: No DNase or RNase activity dected.

is degraded by RNase enzyme, especially if the smear state is limited to the lower part of the gel. The s5 band is not usually distinguished due to its small size.

4-2-3- CDNA synthesis for EGFR gene and its optimization

In reverse transcription reaction for synthesis of cDNA from an mRNA, a primer is needed to specifically or generally bind mRNA to synthesize its tail with an RNA-dependent polymerase enzyme and make a cDNA copy.

1-4-2-3- Contents of cDNA synthesis kit from Takara Company (made in Japan)

2-4-2-3- Kit keeping conditions

The kit should be kept at -20°C freezer.

3-4-2-3-Introducing the Kit

This kit is more suitable for two-step Real-Time PCR (Figure 4-3). The use of this kit is envisaged for both SYBR Green and Taq Man methods, and we proceed based on the former.

4-4-2-3-Steps of the research

Table 5-3: Steps of cDNA synthesis for EGFR gene based on SYBR Green

Reagent	**Volume(μL), RT reaction**
5X primer Script Buffer	2
primer Script RT Enzyme	0.5
Oligo dT primer	0.5
Random Hexamer	0.5
RNase free dH2O	5.5
Total RNA	10

After the solutions and the sample of total RNA were poured into the 0.2 mL microtubule kept on ice according to (Table 5-3), the microtubes were transferred to the thermocycler for incubation based on kit instructions. The incubation was performed in the following conditions:

1. 15 min at 37°C
2. 5 sec at 85°C (inactivation of reverse transcription at high temperature)
3. Rapid transfer to 4°C
4. Keeping at 4°C or -20°C freezer

5-2-3 -CDNA synthesis for miR-212 gene and its optimization

1-5-2-3 -Contents of cDNA synthesis kit for miR-212 gene from Exiqon Company (made in Denmark)

1. 5x reaction buffer

2. Enzyme mix

3. Nuclease water

4. Unisp6, RNA Spike–in–template

2-5-2-3- Kit keeping conditions

The kit should be kept at -15 to -25°C freezer.

3-5-2-3- Introducing the kit

The kit is used for two-step Real-Time PCR.

4-5-2-3-Steps of the research

Table 6-3: Steps of cDNA synthesis for miR-212 based on SYBR Green

Reagent	Volume(μL), RT reaction
5x Reaction buffer	2
Nuclease – free water	4.5
Enzyme mix	1
Synthetic RNA soaking, optionally replace with H2O if omitted	0.5
Template total RNA (5 ng/μL)	2
Total volume	10

1. Total RNA extracted for use in this step should be diluted with nuclease-free water, so that its concentration should reach 5 ng/L.
2. 5xreaction buffer and nuclease-free water were poured into 0.2 mL microtube according to the Table and immediately put on ice.
3. UniSp6 RNA spike-in template can also be employed. First, it should be dissolved in 80 μL of nuclease-free water, mixed thoroughly using vortex to be used as the positive control for the kit; otherwise, it must be used according to the Table and with an equal volume of nuclease-free water. After liquefying the above mentioned primer, it was left on ice for 15-20 minutes. In the meantime, the enzyme mix was immediately taken out of the freezer and added to the contents of the microtubebased on the Table. After 15-20 minutes,UniSp6 RNA spike-in template was poured into the microtube and finally the diluted

RNA was added. All the contents of the microtube were mixed with vortex and spinned down by a refrigerated centrifuge.

4. In the final step, the microtubes must be incubated in the following conditions:

a) 60 min at 42°C for reverse transcription

b) 5 min at 95°C to inactivate reverse transcription at high temperature

c) Rapid transfer to 4°C

d) Keeping at 4°C or -20°C freezer

5-5-2-3- Introducing the kit

1. All solutions should be mixed and homogenized using vortex before testing.
2. The solutions were spinned down after mixing using a refrigerated centrifuge.
3. The resulting cDNA should not be used immediately for Real-Time PCR but must first be kept at refrigerator for a short time.

6-2-3- Real-Time PCR test for EGFR gene

To perform this test, first the Forward and Reverse primers of the genes in question, namely EGFR and the housekeeping (GAPDH)are needed. Therefore, EGFR and GAPDH gene primers were determined using Nishimura (143) and Mohsenzadegan (144) research, respectively and submitted to Cinnagen Company for synthesis.

Table 7-3: Sequence of EGFR and GAPDH gene primers

Name gene	Seq.(5 -3)
Forward EGFR	GGAGAACTGCCAGAAA CTGACC
Reverse EGFR	GCCTGCAGCACACTGGTTG
Forward GAPDH	TTGCCATCAATGACCCCTTCA
Reverse GAPDH	CGCCCCACTTGATTTTGGA

According to the instructions of Cinnagen kit, both primers were dissolved with a certain amount of sterile and nuclease-free water with pH=7as follows:

Primer forward EGFR + 146.4 microliter nuclease free water
Primer reverse EGFR + 170.55 microliter nuclease free water
Primer forward GAPDH + 157.12 microliter nuclease free water
Primer revers GAPDH + 172.03 microliter nuclease free water

With the volume of water that was used, a 100 µM concentration of primerwas prepared, which is generally ten times the nanomolar concentration of oligonucleotide. Then, it was mixed for 5 seconds and if any sediment remained at the bottom of the vial, it was heated for 1-5 minutes at 55°C and thoroughly mixed. In most applications, after preparing a 100 µM concentration, a 10 µM concentration is prepared. For example, 90 µL of nuclease-free water must be added to 10 µM oligonucleotide.

- Sinaclon Company recommendations on water use:

DEPC water as well as water with acidic pH<5 is not recommended because oligonucleotides are gradually degraded under these conditions.
After preparing the primers using Ampliqon kit of Viragen Company (Germany), EGFR and GAPDH gene were prepared for Real-Time PCR test in Roche device as follows (Figure 2-3).

Figure 2-3: Light Cycler *96

1-6-2-3- Contents of the kit

The kit contains Master Mix solution.

2-6-2-3- Kit keeping conditions

The kit must be kept at -20°C.

3-6-2-3- Steps of the research

Since a 0.5 mM concentration of primers in reaction with 20 μL volume is required for Real-Time PCR, 1-μL volume of primers should be used to achieve the desired concentration. The solutions were used according to the Table below. It should be noted that to measure the accuracy of Real-Time PCR, two strips were made from each sample (each sample was used in duplicate) to be able to check for errors.

Table 8-3: Preparation of sample for EGFR gene for Real-Time PCR test

Component	Volume(μL)
Master mix	10
Primer A[1]	1
Primer B[2]	1
Nuclease free water	6
Template DNA	2
Total Volume	20

4-6-2-3 -Kit instructions

1. Adding master mix to the vial must be done in a dark and sterile environment (preferably under the hood).
2. There is no need to vortex the vials.
3. Ampliqon kit performs the two-step Real-Time PCR.

1 Forward primer 10 micromolar (EGFR or GAPDH)
2 Reverse primer 10 micromolar (EGFR or GAPDH)

Table 9-3: Ampliqon kit conditions for EGFR gene for Real-Time PCR reaction

Cycles	Duration of cycle	Temperature
1[1]	15 minutes	95°c
25 - 35	15 – 30 seconds[3] 60 seconds[4]	95°c 55 – 60°c[2]

7-2-3- Real-Time PCR test for miR-212 gene

For this test in which Exiqon kit (made in Denmark) was used, miR-212-3p and miR-212-5p primers as well as miR-103a-3p gene primer (housekeeping) were designed and synthesized by the kit manufacturer in lyophilized form.However, the primer was only miR-103a-3p according to studies.

Table 10-3: Sequence of miR-212 and miR-103a-3p 50 gene primers

Name gene	Seq.(5 -3)
miR-212-3p	UAACAGUCUCCAGUCACGGCC
miR-212-5p	ACCUUGGCUCUAGACUGCUUACU
miR-103a-3p	AGCAGCAUUGUACAGGGCUAUGA

1-7-2-3- Contents of Kit

The kit contains master mix solution.

2-7-2-3- Kit keeping conditions

The kit has to be kept at -20°C.

3-7-2-3- Steps of the research

Table 11-3: Preparation of sample for miR-212 for Real-Time PCR reaction

Component	Volume(μL)
PCR Master mix	5
PCR primer mix	1
Diluted cDNA template	4
Total volume	10

3 For activation of thr TEMPase hot start enzyme

4Choose an appropriate annealing temperature for the primer set used

5 Denaturation time varies between thermocyclers.

6 Set the qPCRinstrument to detect and report fluorescence during the annealing/extension step of each cycle.

1. According to Exiqon kit instructions, 220 μL of nuclease-free water should be added to all the primers to dissolve,after which they are mixedusingvortex and centrifuged with a refrigerated centrifuge.

2. 1:80 concentration of cDNA template should be prepared; for example,395 μL of nuclease free water+ 5 μL of cDNA template

4-7-2-3- Kit instructions

1. Before performing the work and after removing the kit from -20°C freezer, nuclease-free water, PCR Master mix and cDNA should be placed on ice for 15-20 minutes to defreeze.

2. Real-Time PCR should be performed in a dark, sterile environment and on ice.

3. The microtubes should be mixed usingvortex and spinned down with a centrifuge.

After preparation, the samples were transferred to Roche device for analysis and reading.

Table 12-3: Exiqon kit conditions for miR-212 for Real-Time PCR reaction

Polymerase Activation / Denaturation	, 10 min95$^{\circ c}$
40 amplification cycles	, 10 min95$^{\circ c}$, 1 min, ramp – rate 60$^{\circ c}$ 1.6 $^{\circ c/s}$optical read
Melting curve analysis[1]	Yes

3-3- Type of study

This is a fundamental-practical study.

4-3- Study population

Fundamentally, this study is based on previous studies on miRNAs in cancer samples.Considering that this is the first study of its type in the world, the sample size at baseline was 72.Thirty-six cases were infected with T. rubrum and 36 controls were taken from the same patients at a distance of 15-20 cm from the lesion site that was intact and not infected with the fungus.The correlation coefficient of pilot study was 0.5, confidence level 0.95, test power 0.9 based on the following formula taken from the book of Kazem Mohammad:

$$n = \frac{[z_{1-\frac{\alpha}{2}} + z_{1-\beta}]^2}{\left(\frac{1}{2}\ln\frac{1+\rho}{1-\rho}\right)^2} + 3$$

The sample size was 36 based on the above formula.

5-3- Data collection and analysis

There are several methods for data analysis. The Ct parameter used as a figure in calculations is a criterion in Real-Time PCR. An internal standard is employed to eliminate fluctuations in the amount of RNA in the reaction

1Melthing Curve analysis of the PCR product is recommended to verify specificity and identity of the amplification reaction. Melting curve analysis is an step built in to the software of instruments.

as well as performance errors of the device and operator. These standards should be consistently expressed in all tissues, and our experiment should not alter them in comparison to the control sample. For this purpose, GAPDH and miR-103a-3p genes were used as reference genes in this research. The expression of these genes is in a constant leveland comparison between treated sample and internal control can indicate the increase or decrease of their expression. Since the expression of these genes should be the samein all samples, if there is a difference between Cts of these genes in various samples, it means that the test conditions have not been similar for all samples, and the Ct of the desired genes can be corrected according to these genes. Because these genes are essential for life of the cell, they usually have more expression than other genes (lower Ct).

Livak method ($2^{-\Delta\Delta Cq}$) was used to determine the changes in expression of the target gene compared to the internal control gene (GAPDH)as well as miR-103a-3p in skin samples with Tinea compared to normal skin samples (margins).
To normalize the data, the Ct of the studied gene in each sample was subtracted from Ct of the internal control gene of the same sample. Since the normal data for each sample is obtained by subtracting Ct of the gene in question from that of the internal control gene, the normalized data is called ΔCt:

A)The mean Ct of Tinea lesions was subtracted from mean Ct of the reference Tinea lesion (ΔCt of Tinea lesion).

B)Mean Ct value of the control sample was subtracted from that of the reference control (ΔCt of control).

C)ΔCt value of the Tinea lesion is subtracted from ΔCt value of the control. The new parameter obtained by subtracting ΔCt of the desired gene in Tinea lesion from ΔCt of the control sample is called normalized data (ΔΔCt).

When two or more groups are relatively compared,fold change is the best way to express the testresult.

Since lowerΔΔCt value means higher expression (i.e.fold change), ΔΔCt must first be multiplied in -1, and at this point, it can be statedthat higher-ΔΔCtis the logarithmicfold change (base 2) because each cycle of Real-Time PCR doubles the product. To be able to linearize -ΔΔCt, it must be written as $2^{-\Delta\Delta Ct}$that would represent the fold change for each sample. Therefore, the changes in the expression of target genes was calculated using$2^{-\Delta\Delta Ct}$formula (145).
Gene expression was calculated by Microsoft Excel using $2^{-\Delta\Delta Ct}$formula, which is an accurate method for measuring gene expression in Real-Time PCR.

6-3- Statistical analysis method

Quantitative data were described by calculating mean and standard deviation and qualitative or categorized data were assessed by frequency distribution tables. For data analysis, the tests related to correlation coefficients and, if necessary, paired tests were used considering ***P*** value<0.05. Software related to Real-Time PCR data analysis was also used. Statistical analysis was conducted using SPSS.21 software with 95% confidence interval.

7-3- Ethical principles

A written consent form was used, which was filled with the permission of the participants whose specifications remained completely confidential.

8-3- Ethics code

The ethics code of this research was IR.KMU.REC.1394.416.

9-3- Place and time of the study

The study was conducted in Kerman University of Medical Sciences over a period of one year.

10-3- Problems and limitations

1. Due to exchange rate fluctuations for consumables of molecular studies, the problems associated with this issue were not predictable.
2. The study period was prolonged because this technique was set up for the first time in Iran.

CHAPTER IV

RESULTS

Summary of sample collection

After taking skin samples of patients referred to Razi Dermatology Hospital in Tehran from fungus-infected as well as seemingly healthy margins of lesions as stated in Introduction, the samples were poured into cryotubes and placed in -70°C for molecular tests. Some of the lesion flakes of participant shaving a higher chance of T. rubrum due to clinical signs were culturedas slide culture in Sabouraud dextrose agar medium. Sampling and culture continued until 36 samples of T. rubrum were obtained using the culture resultsas well as microscopic observation of slide and coverslip from slide culture.

1-4-Culture results from skin samples

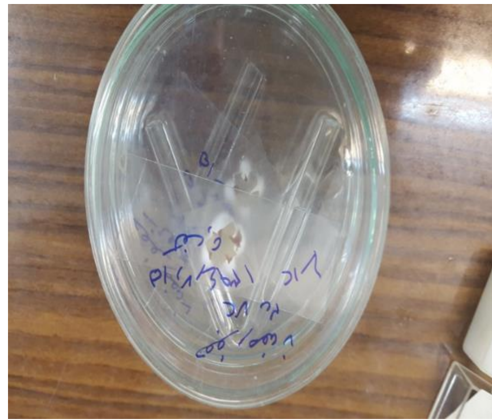
After slide cultureof flakes suspicious of Tinea on Sabouraud dextrose agar medium, the following results were obtained after about two weeks' growth of T. rubrum colonies, which was in accordance with the macroscopic characteristics discussed in the Introduction. As shown in the below Figure, the flat or convex colonies with cotton, villous to fluffy appearance in white to red colors were grown. The underside of the culture medium in Figure 4-1 is pinkish due to the freshness of the colony, which became redder over time until it turned brown in older cultures, which is one of the helpful diagnostic methods for this fungus, (Figure 1-4).

Kaminski's dermatophyte identification scheme is sometimes used for precise detection of T. rubrum, which uses special media such as Sabouraud dextrose agar containing 5% salt,peptone agar, Littman Oxgall agar or lactritmel agar (3).As the mentioned media were not available to us, only Sabouraud dextrose agar medium containing cyclohexamide and chloramphenicol was used. For differential diagnosis of T. rubrum from T. mentagrophytes, Corn Mealdextrose agar medium was used in which T. rubrum was seen in red-purple color.

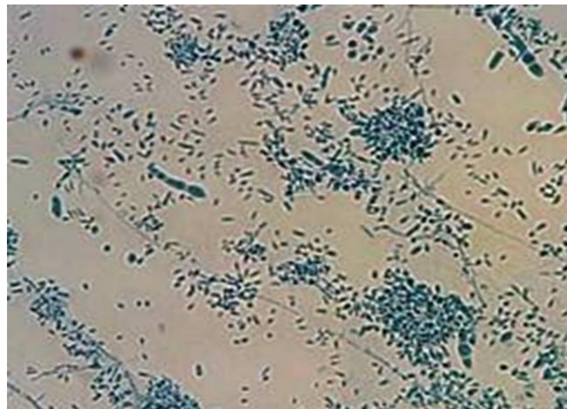
Figure 1-4: T. rubrum colony isolated from patients with Tinea

2-4- Slide culture results of T. rubrum colonies

To confirm the presence of the desired fungus, culture slides were prepared from all samples of Tinea lesions, which is described in detail in the Introduction. After preparing the slide culture for all samples of Tinea separately (part A of Figure 2-4) and leaving the fungus to grow for one week, the culture medium between the slide and coverslip was discarded. Lactophenol cotton blue was then poured on each of the slides and coverslips,was covered with another slide and coverslip and examined separately for the presence of fungal reproductive structure under a microscope with 40× magnification.Given the high sensitivity of the fungus' reproductive structure,extreme care was taken to not damage the slide and coverslip and the presence of T. rubrum was confirmed as shown in part B of Figure 2-4based on the following explanations. As we know, the fungi isolated from chronic lesions usually lack microconidia or macroconidia, but those isolated from inflammatory lesions have abundant macroconidia with a colony similar to T. violaceum. In (part B of Figure 2-4), which was separated from an inflamed lesion, the mycelia are colorless, transparent, delicate, and transversely walled with slender, pencil-shaped, elongated or sausage-shaped microconidia. The wall of the macroconidia is smooth, the macroconidia are parallel to each other and have 2-8 middle blades but the number of macroconidia is limited.Microconidia are lateral and scattered on the surface of the slide in spherical, teardrop or pear shapes or like wooden nails around the mycelia, which were seen in large numbers in this Figure. Racket-shaped hyphae and chlamydoconidia were not observed in this Figure because the culture was fresh, which may be seen in older samples. It should be noted that the formation of macroconidia is intensified in blood agar-based medium (Difco) (3), which was not used in this study due to lack of culture medium.



(A)



(B)

Figure 2-4: (A) Preparation of culture slides and (B) Microscopic view of T. rubrum with ×40 magnification with lactophenol cotton blue staining

3-4- Results of maintaining the quality and quantity of total extracted RNA

In this study, after extracting total cellular RNA from each of the clinical specimens of T. corporis and the seemingly normal margin of the lesion that was prepared under RNase free conditions, the concentration of each sample was determined using Nanodrop. Also, A260/A280 ratio was calculated for each sample and the values obtained for the samples were read between 1.7 and 1.9 by the device, which indicated the acceptable purity of the extracted RNA and its low contamination with protein and genomic DNA.
To determine the quality and keep the integrity of extracted RNA from each sample, a small amount of RNA from each sample was run on 1% agarose gel and electrophoresed. At this stage, it was found that the extracted whole RNAs had maintained their integrity and that no RNA fragmentation had occurred in the process of separating the sample from the patient as well as during extraction. It is noteworthy that a gentle and uniform smear was seen from top to bottom of the gel due to the presence of different mRNA transcripts in the extracted total RNA;moreover,S28, S18 bands and S5 ribosomal RNA were visibly observed in each sample. Figure 3-4 shows the extracted total RNA electrophoresis image of a sample.

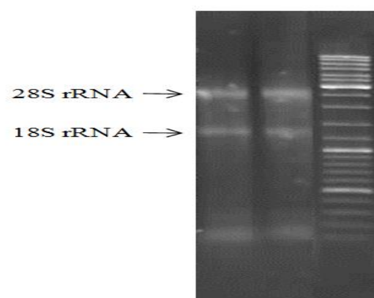


Figure 3-4: Image of 1% agarose gel from total RNA extracted from clinical samples

4-4- Analysis of the results of changing expressions of miR-212 and EGFR genes

1-4-4 -Analysis of the results of miR-212 gene expression changes

According to what mentioned in Materials and Methods concerning the method of calculating the expression of miR-212 genes and its internal control gene (miR-103a-3p) in the samples of this study, based on the data obtained from mean ΔCt value (i.e. the Ct value oftar get miR-212 geneminus Ct value of internal control miR-103a-3p gene),it can be concluded that ΔCt of miR-212 gene in Tinea lesions had significantly changed in comparison to the healthy marginal tissue (***P*** value ≤ 0.0001), so that ΔCt in Tinea lesion sample was 5.77±0.316, which was lower relative to the healthy margin tissue (9.86±0.155).Since the Ct value is inversely correlated with the copy number of gene, miR-212 gene expression was higher in Tinea lesions than in healthy marginal tissue, (Table 1-4).

Table 1-4: Comparison of mean ΔCt changes in miR-212 gene expression in Tinea samples and healthy margin tissue

P **value**	**SD**	**Mean**	**miR-212 gene expression**
0.0001	0.316268	5.77308	miR-212 of Tinea lesion
	0.155985	9.86417	miR-212 of healthy margin

2-4-4-Analysis of EGFR gene expression change results

Analysis of t-test results on the expression of EGFR and GAPDH genes in Tinea lesion compared to healthy margin tissue,namely mean ΔCt value (Ct value of EGFR target gene minusthat of GAPDH internal control gene) showed a significant difference (***P*** value ≤ 0.0001). ΔCt in the sample of Tinea lesion and healthy marginal tissue was 9.12±0.255 and 6.53±0.277, respectively.Because Ct value is inversely related to the copy number of gene, the expression of EGFR gene in Tinea lesion wasless than healthy margin tissue, (Table 2-4).

Table 2-4: Comparison of mean changes in ΔCt EGFR gene expression in Tinea samples and healthy margin tissue

P **value**	**SD**	**Mean**	**EGFR gene expression**
0.0001	0.255136	9.12096	EGFR of Tinea lesion
	0.277011	6.53223	EGFR of healthy margin

3-4-4- Analysis of results of simultaneous expression changes of miR-212 and EGFR genes

The important point in comparing the expression of miR-212 and EGFR as target genes is that the ΔCt value of EGFR gene was higher than miR-212 in Tinea lesion, which was statistically significant (***P*** value ≤ 0.037) and hence the expression of EGFR gene in Tinea lesionwasless than that of healthy marginal tissue. Also, the results of data analysis based on the formula $2^{-\Delta\Delta Ct}$ also expressed the same changes as shown in, (Table 3-4).

Table 3-4 Simultaneous comparison of changing miR-212 and EGFR gene expressions

P **value**	**SD**	**Mean**	**Expression of target genes in Tinea lesion**
0.037	0.313268	5.77308	miR-212 of Tinea lesion
	0.255136	9.120906	EGFR of Tinea lesion

5-4-Results of Real-Time PCR reactions in the form of amplification and melting curves

After performing DNase treatment reactions and cDNA synthesis as described in Chapter 3 (Materials and Methods) of this thesis, Real-Time PCR reactions of target genes were conducted in duplicate for all samples (test and control). The amplification and melting curves of internal control and reference genes used in this study (respectively GAPDH and miR-103a-3p) along with the genes in question are presented below, which are EGFR and miR-212 (Figures 4-4 to Figure 7-4).

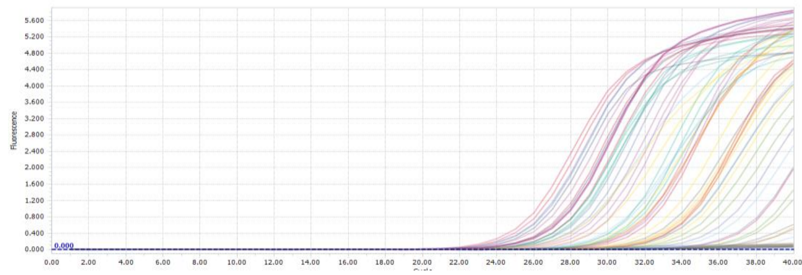


Figure 4-4: EGFR gene amplification curve and GAPDH internal control

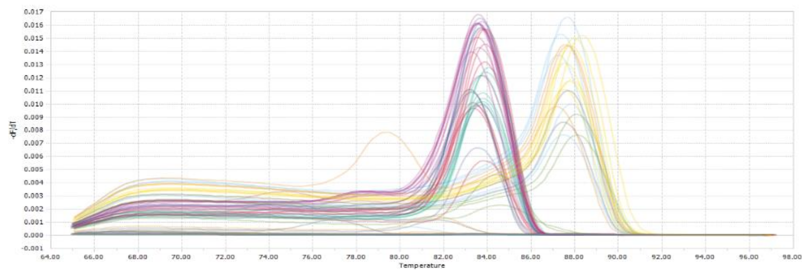


Figure 5-4: Melting curve of EGFR genes and GAPDH internal control

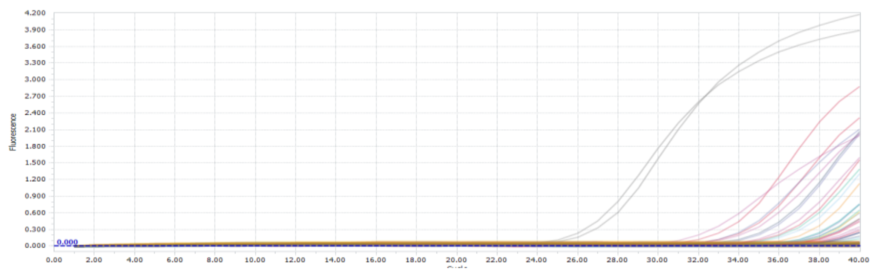


Figure 6-4: MiR-212 gene amplification curve and miR-103a-3p internal control

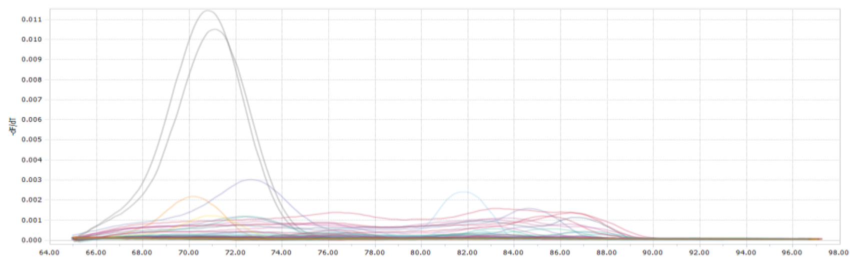


Figure 7-4: Melting curve of miR-212 genes and internal control of miR-103a-3p

After analyzing the expression changes in EGFR and miR-212 genes **with their internal control genes** (GAPDH and miR-103a-3p), the results are given in sections 1-4-4, 2-4-4 and 3-4-4 and calculated using Excel software as shown in the below diagram.

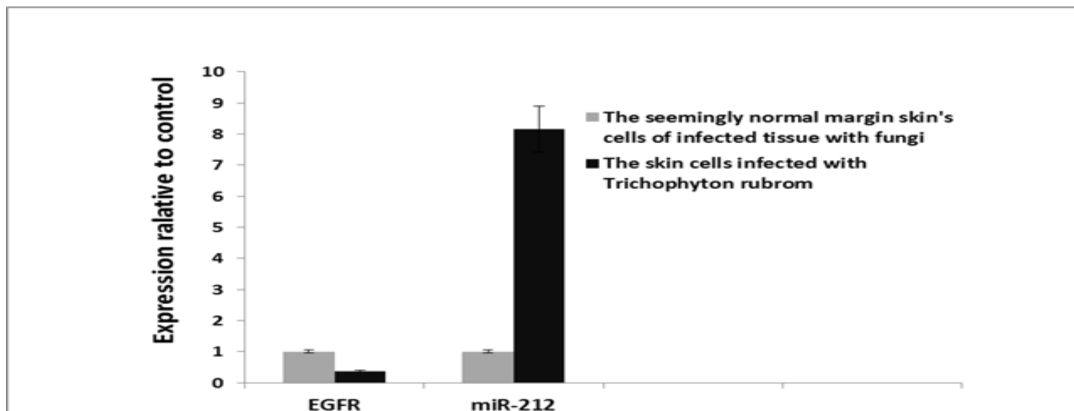


Diagram 1-4: Expression of EGFR and miR-212 genes in skin cells infected with T. rubrum and seemingly normal tissue around the lesions

CHAPTER V

DISCUSSION, CONCLUSION AND SUGGESTIONS

The importance of project

The infection with dermatophytesleading to various types of Tinea is widespread in the world, including Iran.In the mean time, T. rubrum dermatophyte accounts for a high percentage of this infection.Initial diagnosis of dermtophytosis by T. rubrum using direct slide preparation and observation under a microscope is subject to culture of the fungus because of its similarity to T. mentagrophytes, which is time consuming and delays the patient test result for at least 2-3 weeks. Therefore, we decided to take advantage of modern diagnostic method susing miRNAs in this regard to achieve promising results both in the diagnosis and likely treatment of dermatophytosis(especiallyT. rubrum) in near future.

As explained below, the presence of EGFR gene as an epidermal growth factor receptor has been shown to prevent the presence and colonization of dermatophytes on human skin. Inhibition of EGFR gene when confronting the dermatophyte leads to dermatophytosis and will have a direct effect on the presence or absence of AMPs in dermatophyte-infected keratinocytes. EGFR-related AMPs that help the host control and inhibit fungal growth or lead to disease development have been reported in research on hBD-3 and RNase7.

On the other hand, the role of miRNAs as regulatory RNAs silencing EGFR gene expression by interfering with the expression of target gene mRNA has been proven (105).

There has been extensive research on the effect of negative induction of miRNAs on EGFR gene resulting in the silencing of this gene and the development of various diseases, especially in the field of cancer.Studies by Bernhard at International Cancer Institute in the United States on head and neck squamous cell carcinoma (HNSCC) indicates that increasing expression of miR-212 suppresses EGFR mRNAs and eventually reduces EGFR expression (112).In his study of 384 miRNAs associated with GFR gene, miR-212 had the highest relative expression with the gene in question, as shown in (Table 1-5). The researcher concluded that increasing EGFR gene expression would greatly reduce the incidence of HNSCC, which could be beneficial to cancer patients receiving chemotherapy or radiotherapy (146-148). This study was the first one to show the important role of miRNAs in the regulation of gene receptor ligands. Later, the miRNA regulations in cancers were found to be important and it was shown that gene receptor ligands are regulated by miRNAs that have been reported before this research (149-151).

Bernhard concluded that miR-212 had a specific regulatory role on EGFR and detected EGFR gene levels at different cells afflicted with HNSCC. Decreased expression of miR-212 gene was identified by increasing expression of EGFR gene (152). Therefore, in the mentioned study, the relationship between miR-212 and EGFR gene was clearly indicated.

Another study by Yasemin Helene Firat explained that the presence or absence of AMPs in keratinocytes was affected by EGFR gene expression in the skin and the confrontation mode with skin infections. The most important defense of keratinocytes against microorganisms is to increase the expression of AMPs (28, 32, 69). In herinvestigation, it was found that keratinocytes were the first cells to encounter the common dermatophyte T. rubrum (23, 70). The researcher synthetically produced IFN-γ and IL-17A cytokines, which increase the presence of AMPs in keratinocytes (153). Finally, it was found that AMPs of keratinocytes, including HBD-3 and RNase7, had the ability to counteract T. rubrum (36). Since the inhibition of EGFR gene will adversely affect the presence of AMPs, it often leads to the establishment of dermatophytes in the skin, which will lead to Tinea (60).

1-5- Discussion and Interpretation

As mentioned in the importance of project section, previous research and in particular the study of Bernhard and Yasemin Helene Firatare related to our discussion, namely the function of miR-212 and EGFR genes and their relationship with AMPs with interesting results cited at the beginning of this chapter. We also attempted to conduct research on thetopic and generalize this interesting finding to other diseases, including dermatophytoses, so that a new chapter can be opened in this field that leads to further research on the interaction between non-coding RNAs with fungi of different species and genera. Therefore, after collecting tissue samples infected with T. rubrum and healthy tissue samples from the same patient by observing the mentioned conditions as the control sample,RNA isolation and optimization tests were followed by DNA synthesis and optimization as detailed in Materials and Methods using an accurate and sensitive Real-Time PCR method. EGFR and mRNA genes closely associated with the mentioned gene were quantified based on MiRWalk database from which miR-212 was the chosen candidate. Finally, using bioinformatics analysis as stated in the analysis of results section, acceptable results were achieved in this field.

1-1-5- Discussion on increasing or decreasing expression of miR-212 gene

According to (Diagram 1-4) that was referred to in Results, the expression of miR-212 gene in skin cells changed after they became infected with T. rubrum. In fungal lesion flakes, there is an increase in the expression of miR-212 but it is decreased inhealthy skin cellsof the lesion margin. As mentioned in the Introduction, miRNAs control the expression of their target genes by acting on mRNA of target genes.Nevertheless, it should be noted that a particular miRNA molecule does not have only one target mRNA; in other words, a single miRNA has more than

one target mRNA molecule. The large number of miR-212 target mRNAs that are increased or decreased due to a respective decrease or increase in their expression within keratinocytes requires further studies that has not been the subject of this study. The reverse is also true, as any mRNA molecule can affect a miRNA. In the present study, miR-212 is no exception to this general rule, so that the changing expression of miR-212 following infection of skin cells with T. rubrum can be attributed to this issue.More broadly, when the desired cells in this study, namely skin cells, became infected with T. rubrum, an infectious shock was actually inflicted on these cells following encounter with the fungus. In the interaction between skin cells and the fungal agent, these cells may express different levels of miR-212 to activate various genes they need to express.For example,what level of miR-212 gene is expressed at the beginning of a fungal infection and to what extent it affects the expression of EGFR gene, what extent this increase of miR-212 expression continues during disease progression, whether this increasing miR-212 expressionis multiplied in the acute phase of the infection or is there a threshold for it that doses not surpass a specific level.Another hypothesis is that whether with the improvement of the infection and the consumption of drugs mainly in the form of topical antifungal agents there will be a decrease in the expression of miR-212 gene andif this decrease in gene expression occurs rapidly or at a slow rate.Obviously, the assumption of reduced expression is more likely because we know that the treatment of fungal diseases will be time consuming and sometimes tedious for the patient.We don't know whether the expression of this gene reaches the level before infection with the fungus orthere is partial gene expression at the scar of Tinea lesion. Another question is the effect of the number of fungal lesions on increasing expression of miR-212 gene; in other words,to what extent the pathological and physiological conditions of the disease increase the gene expression that is not the subject of this research and requires more extensive research. It is also important to note that a large number of miR-212 molecular targets in different cells have not been demonstrated in laboratory in different cells, including skin cells under different physiological and pathological conditions. Many of these targets have been predicted with the help of bioinformatics software that may be suitable for the miR-212, which has complicated the study of miR-212 molecular targets. Therefore, it seems that a proper solution to elucidate the effect of miR-212 for different molecular targets in the cell is the application of high-throughput tests (HTP) such as microarray and next-generation sequencing (NGS).

2-1-5-Discussion on increasing or decreasing expression of EGFR gene

As discussed on miRNA, EGFR as the target gene may be affected by several miRNAs simultaneously and show different expression changes;however, due to the present discussion, only the relationship of expression with miR-212 has been evaluated and thus the interpretation of its expression changes according to the findings of the fourth chapter of this study is as follows. According to(Figure 1-4), the expression level of EGFR gene was altered in skin cells after their infection with T. rubrum. In shavings with fungal lesions, the expression of EGFR gene is decreased but it is increased in healthy skin cells in the margin of lesion. One of the arguments about this gene is perhaps the rate of decrease in EGFR gene expression during the maturation of the diseasewhen the fungus has reached its maximum function in skin cells and demonstrated severe symptoms of the disease in vesicular form and severe scaling of T. corporis.

3-1-5 -Discussion on simultaneous change of EGFR and miR-212 genes

As shown in (Diagram 1-4), concomitant changes in EGFR and miR-212 genes in T. rubrum-infected skin cells appear in the form of increasing miR-212 and decreasing EGFR expression, which is reversed in healthy skin cells around the fungal lesion. One of the debates that can be presented in this section is whether the increase in miR-212 expression and decrease in EGFR expression of fungal lesions is constant in all individuals with any genetics or differs among different races and genders.Perhaps underlying diseases such as diabetes or autoimmune diseases play a role in the expression of genes, which requires extensive research. It is hoped to do research on these topics in future.

2-5-Conclusion

According to the mentionedstudies, the colonization and activity of T. rubrum in skin cells is dependent on the presence or absence of EGFR-dependent AMPs, so that the absence of specific AMPs can lead to dermatophyte colonization (T. rubrum in our research) and result in Tinea development in the patient,which is associated with miR-212-dependent decrease in the expression of EGFR gene and directly affects mRNA of EGFR gene and silences its expression. Bioinformatics analyses of the findings according to Diagram 4-1 showed that the expression of EGFR gene in skin cells inhibits infection with Tinea. On the other hand, the mechanism of action of microRNA is such that it silences gene expression; therefore, bioinformatics studies indicated that miR-212 can affect EGFR as a potential target in these cells as well, although proof of this requires functional studies.MiR-212 in tissue samples infected with T. rubrum dermatophyte significantly reduced the expression of EGFR gene and increased the expression of miR-212 gene about 8-foldrelative to the expression of EGFR gene. On the other hand, as shown in the diagram, the expression of miR-212 is much lower than that of EGFR gene;therefore, due to the presence of EGFR gene and consequent existence of mentioned AMPs in the Introduction, the patient has been able to resist the invasion of T. rubrum without developing Tinea. However, it is possible that because of intercellular protein connections, increasing expression of miR-212 gene could be transferred from the infected tissue to healthy parts of patient's skin and the person will suffer from wider and deeper lesions in the future if not diagnosed and treated on time.

3-5-Suggestions

According to the description of the results of this study, the use of miR-212 for diagnostic or therapeutic applications has certain considerations that require further research. As we know, a molecule can be used as a biomarkerwith diagnostic or therapeutic value when ithas high sensitivity and specificity and the expression of non-coding RNAs (whose product can be protein or any other compound) shows a significant difference between two different treatments in healthy and morbid conditions. Based on the above statements and the results of this study, it can be claimed that further studies on the effect of T. rubrum in different cells are needed to determine whether the degree of expression deviation is appropriate with regard to the two abovementioned requirements. Changing the optimal concentration of fungal treatments and also considering the genome of human skin cells is among these studies. For instance, some patients have specific genetic traits in their genome that make different their confrontation with fungal infections(including T. rubrum) than others, or some people are more quickly infected with a lower dose offungi even in the absence of controllable underlying factors such as the use of immunosuppressive drugs. This difference is due to the changing expression of different genes of these individuals in response to T. rubrum infection. Different types of non-coding RNAs such as miRNAs are among these genes because as we know these miRNAs are key molecules regulating the expression of genes in physiological and pathological processes in cells.Therefore, miR-212 may also be involved in the response of skin cells to T. rubrum. The extent to which cells are affected by miR-212 after infection with T. rubrum and the mechanism of this role is not yet understood and further studies are needed in this respect.

1. Functional study of pre-expression or suppression of miR-212 and EGFR genes in cell cultures infected with Trichophyton rubrum

2. Study of miR-212 and EGFR simultaneously and separately in other clinical specimens that may be infected with this fungus.

3. Study of the effect of miR-212 suppression on EGFR pre-expression in the process of fungal progression under cell culture (study of the effect of treatment).

REFERENCES

1. Havlickova B, Czaika VA, Friedrich M. Epidemiological trends in skin mycoses worldwide. Mycoses. 2008;51(s4):2-15.
2. Seebacher C, Bouchara J-P, Mignon B. Updates on the epidemiology of dermatophyte infections. Mycopathologia. 2008;166(5-6):335-52.
3. Amir Y. Medical mycology Cutaneaus Mycoses (Dermatophytosis). Iran University Of Medical Sciences: 1; 1387. 87 p.
4. Dahdah MJ, Scher RK. Dermatophytes. Current Fungal Infection Reports. 2008;2(2):81-6.
5. Brasch J. Pathogenesis of tinea. JDDG: Journal der Deutschen Dermatologischen Gesellschaft. 2010;8(10):780-6.
6. Nenoff P, Krüger C, Ginter-Hanselmayer G, Tietz HJ. Mycology–an update. Part 1: Dermatomycoses: causative agents, epidemiology and pathogenesis. JDDG: Journal der Deutschen Dermatologischen Gesellschaft. 2014;12(3):188-210.
7. Maraki S, Tselentis Y. Dermatophytoses in Crete, Greece, between 1992 and 1996. Mycoses. 1998;41(3-4):175-8.
8. Maraki S. Epidemiology of dermatophytoses in Crete, Greece between 2004 and 2010. Giornale italiano didermatologia e venereologia: organo ufficiale, Societa italiana di dermatologia e sifilografia. 2012;147(3):315-9.
9. Devliotou-Panagiotidou D, Koussidou-Eremondi T, Badillet G. Dermatophytosis in northern Greece during the decade 1981–1990. Mycoses. 1995;38(3-4):151-7.
10. Tsoumani M, Jelastopulu E, Bartzavali C, Vamvakopoulou S, Dimitracopoulos G, Anastassiou E, et al. Changes of dermatophytoses in southwestern Greece: an 18-year survey. Mycopathologia. 2011;172(1):63-7
11. Maraki S, Mavromanolaki VE. Epidemiology of Dermatophytoses in Crete, Greece. *Med Mycol J*. 2016;57(4):E69-E75.
12. Maraki S, Nioti E, Mantadakis E, Tselentis Y. A 7-year survey of dermatophytoses in Crete, Greece. Mycoses. 2007;50(6):481-4.
13. Burzykowski T, Molenberghs G, Abeck D, Haneke E, Hay R, Katsambas A, et al. High prevalence of foot diseases in Europe: results of the Achilles Project .Mycoses. 2003;46(11-12):496-505.
14. Lin RL, Szepietowski JC, Schwartz RA. Tinea faciei, an often deceptive facial eruption. International journal of dermatology. 2004;43(6):437-40.
15. Nicola A, Laura A, Natalia A, Monica P. A 20-year survey of tinea faciei. Mycoses. 2010;53(6):504-8.
16. del Boz J, Crespo V, de Troya M. Pediatric Tinea Faciei in Southern Spain: A 30-Year Survey. Pediatric dermatology. 2012;29(3):249-53.
17. Kieliger S, Glatz M, Cozzio A, Bosshard P. Tinea capitis and tinea faciei in the Zurich area–an 8-year survey of trends in the epidemiology and treatment patterns. Journal of the European Academy of Dermatology and Venereology. 2015;29(8):1524-9.
18. Aghamirian MR, Ghiasian SA. Dermatophytoses in outpatients attending the dermatology center of Avicenna Hospital in Qazvin, Iran. Mycoses. 2008;51(2):155-60.
19. Drakensjö IT, Chryssanthou E. Epidemiology of dermatophyte infections in Stockholm, Sweden: a retrospective study from 2005–2009. Medical mycology. 2011;49(5):484-8.
20. KHOSRAVI A, KORDBACHEH P, BOKAEE S. An epidemiological approach to the zoophilic dermatophytoses in Iran. Medical Journal of The Islamic Republic of Iran (MJIRI). 1994;7(4):253-7.
21. Gibas C, Sigler L, Summerbell R, Hofstader S, Gupta A. Arachnomyces kanei (anamorph Onychocola kanei) sp. nov., from human nails. Medical mycology. 2002;40(6):573-80.
22. DiSalvo AF. Occupational mycoses: Williams & Wilkins; 1983.
23. Weitzman I, Summerbell RC. The dermatophytes. Clinical microbiology reviews. 1995;8(2):240-59.
24. Gräser Y, Kühnisch J, Presber W. Molecular markers reveal exclusively clonal reproduction in Trichophyton rubrum. Journal of clinical microbiology. 1999;37(11):3713-7.
25. F.R.C.V.S WW. The Principles and Practice of Veterinary Surgery Newed.,rev ed. cdl; americana New York , WR.Jenkins; 1894.
26. Gräser Y, Scott J, Summerbell R. The new species concept in dermatophytes—a polyphasic approach. Mycopathologia. 2008;166(5-6):239.
27. Makimura K, Tamura Y, Mochizuki T, Hasegawa A, Tajiri Y, Hanazawa R, et al. Phylogenetic classification and species identification of dermatophyte strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. Journal of clinical microbiology. 1999;37(4):920-4.
28. Harder J, Schröder JM, Gläser R. The skin surface as antimicrobial barrier: present concepts and future outlooks. Experimental dermatology. 2013;22(1):1-5.
29. Hancock RE, Diamond G. The role of cationic antimicrobial peptides in innate host defences. Trends in microbiology. 2000;8(9):402-10.
30. Zasloff M. Antimicrobial peptides of multicellular organisms. nature. 2002;415(6870):389-95.
31. Mangoni ML. Host-defense peptides: from biology to therapeutic strategies. Cellular and Molecular Life Sciences. 2011;68(13):2157-9.
32. Harder J, Schröder J-M. Antimicrobial peptides in human skin. Mechanisms of Epithelial Defense. 86: Karger Publishers; 2005. p. 22-41.
33. Gläser R, Becker K, Von Eiff C, Meyer-Hoffert U, Harder J. Decreased susceptibility of Staphylococcus aureus small-colony variants toward human antimicrobial peptides. Journal of Investigative Dermatology. 2014;134(9):2347-50.
34. Firat YH, Simanski M, Rademacher F, Schröder L, Brasch J, Harder J. Infection of keratinocytes with Trichophytum rubrum induces epidermal growth factor-dependent RNase 7 and human beta-defensin-3 expression. PloS one. 2014;9(4):e93941.

35. Brasch J, Mörig A, Neumann B, Proksch E. Expression of antimicrobial peptides and toll-like receptors is increased in tinea and pityriasis versicolor. Mycoses. 2014;57(3):147-52
36. Fritz P, Beck-Jendroschek V, Brasch J. Inhibition of dermatophytes by the antimicrobial peptides human β-defensin-2, ribonuclease 7 and psoriasin. Medical mycology. 2012;50(6):579-84.
37. Ishikawa T, Kanda N, Hau CS, Tada Y, Watanabe S. Histamine induces human β-defensin-3 production in human keratinocytes. Journal of dermatological science. 2009;56(2):121-7.
38. Feingold KR. Lamellar bodies: the key to cutaneous barrier function. Journal of Investigative Dermatology. 2012;132(8):1951-3.
39. Percoco G, Merle C, Jaouen T, Ramdani Y, Bénard M, Hillion M, et al. Antimicrobial peptides and pro-inflammatory cytokines are differentially regulated across epidermal layers following bacterial stimuli. Experimental dermatology. 2013;22(12):800-6.
40. Wittersheim M, Cordes J, Meyer-Hoffert U, Harder J, Hedderich J, Gläser R. Differential expression and in vivo secretion of the antimicrobial peptides psoriasin (S100A7), RNase 7, human beta-defensin-2 and-3 in healthy human skin. Experimental dermatology. 2013;22(5):364-6.
41. Gallo RL. The birth of innate immunity. Experimental dermatology. 2013;22(8):517.-
42. Do N, Weindl G, Grohmann L, Salwiczek M, Koksch B, Korting HC, et al. Cationic membrane-active peptides–anticancer and antifungal activity aswell as penetration into human skin. Experimental dermatology. 2014;23(5):326-31.
43. Simanski M, Gläser R, Harder J. Human skin engages different epidermal layers to provide distinct innate defense mechanisms. Experimental dermatology. 2014;23(4):230-1.
44. Braff MH, Zaiou M, Fierer J, Nizet V, Gallo RL. Keratinocyte production of cathelicidin provides direct activity against bacterial skin pathogens. Infection and immunity. 2005;73(10):6771-81.
45. Braff MH, Di Nardo A, Gallo RL. Keratinocytes store the antimicrobial peptide cathelicidin in lamellar bodies. Journal of investigative dermatology. 2005;124(2):394-400.
46. Aberg KM, Man M-Q, Gallo RL, Ganz T, Crumrine D, Brown BE, et al. Co-regulation and interdependence of the mammalian epidermal permeability and antimicrobial barriers. Journal of Investigative Dermatology. 2008;128(4):917-25.
47. Gudmundsson GH, Agerberth B. Neutrophil antibacterial peptides, multifunctional effector molecules in the mammalian immune system. Journal of immunological methods. 1999;232(1):45-54.
48. Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L, et al. The human antimicrobial and chemotactic peptides LL-37 and α-defensins are expressed by specific lymphocyte and monocyte populations. Blood. 2000;96(9):3086-93.
49. Di Nardo A, Vitiello A, Gallo RL. Cutting edge: mast cell antimicrobial activity is mediated by expression of cathelicidin antimicrobial peptide. The Journal of Immunology. 2003;170(5):2274-8.
50. Mangoni M. Temporins, anti-infective peptides with expanding properties. Cellular and molecular life sciences. 2006;63(9):1060-9.
51. Conlon JM, Iwamuro S, King JD. Dermal cytolytic peptides and the system of innate immunity in anurans. Annals of the New York Academy of Sciences. 2009;1163(1):75-82.
52. Pask JD, Cary TL, Rollins-Smith LA. Skin peptides protect juvenile leopard frogs (Rana pipiens) against chytridiomycosis. Journal of Experimental Biology. 2013;216(15):2908-16.
53. Arteaga C, editor Targeting HER1/EGFR: a molecular approach to cancer therapy .Seminars in oncology; 2003: Elsevier.
54. Burgess AW. EGFR family: structure physiology signalling and therapeutic targets. Growth Factors. 2008;26(5):263-74.
55. Han W, Lo H-W. Landscape of EGFR signaling network in human cancers: biology and therapeutic response in relation to receptor subcellular locations. Cancer letters. 2012;318(2):124-34.
56. Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. New England Journal of Medicine. 2008;358(11):1160-74.
57. Wheeler DL, Dunn EF, Harari PM. Understanding resistance to EGFR inhibitors—impact on future treatment strategies. Nature reviews Clinical oncology. 2010;7(9):493-507.
58. Ohashi K, Maruvka YE, Michor F, Pao W. Epidermal growth factor receptor tyrosine kinase inhibitor–resistant disease .Journal of Clinical Oncology. 2013;31(8):1070-80.
59. Harris RC, Chung E, Coffey RJ. EGF receptor ligands. The EGF receptor family biologic mechanisms and role in cancer Elsevier, California. 2004:3-14.
60. Eilers Jr R, Gandhi M, Patel J, Mulcahy M, Agulnik M, Hensing T, et al. Dermatologic infections in cancer patients treated with epidermal growth factor receptor inhibitor therapy. Journal of the National Cancer Institute. 2010;102(1):47-53.
61. Rusch V, Klimstra D, Venkatraman E, Pisters P, LangenfeldJ, Dmitrovsky E. Overexpression of the epidermal growth factor receptor and its ligand transforming growth factor alpha is frequent in resectable non-small cell lung cancer but does not predict tumor progression. Clinical Cancer Research. 1997;3(4):515-22.
62. Brabender J, Danenberg KD, Metzger R, Schneider PM, Park J, Salonga D, et al. Epidermal growth factor receptor and HER2-neu mRNA expression in non-small cell lung cancer is correlated with survival. Clinical Cancer Research. 2001;7(7):1850-5.
63. Ekstrand AJ, James CD, Cavenee WK, Seliger B, Pettersson RF, Collins VP. Genes for epidermal growth factor receptor, transforming growth factor α, and epidermal growth factor and their expression in human gliomas in vivo. Cancer research. 1991;51(8):2164-72.
64. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Critical reviews in oncology/hematology. 1995;19(3):183-232.

65. Chow N, Liu H, Lee E, Chang C, Chan S, Cheng H, et al. Significance of urinary epidermal growth factor and its receptor expression in human bladder cancer. Anticancer research. 1997;17(2B):1293-6.
66. Ueda S, Ogata S, Tsuda H, Kawarabayashi N, Kimura M, Sugiura Y, et al. The correlation between cytoplasmicoverexpression of epidermal growth factor receptor and tumor aggressiveness: poor prognosis in patients with pancreatic ductal adenocarcinoma. Pancreas. 2004;29(1):e1-e8.
67. King CR, Kraus MH, Aaronson SA. Amplification of a novel v-erbB-related gene ina human mammary carcinoma. Science. 1985;229:974-7.
68. Grandis JR, Sok JC. Signaling through the epidermal growth factor receptor during the development of malignancy. Pharmacology & therapeutics. 2004;102(1):37-46.
69. Schröder J-M. Antimicrobial peptides in healthy skin and atopic dermatitis. Allergology International. 2011;60(1):17-24.
70. Smijs TG, Pavel S. The susceptibility of dermatophytes to photodynamic treatment with special focus on Trichophyton rubrum. Photochemistry and photobiology. 2011;87(1):2-13
71. Larone D. Yeast and yeast-like organisms, p 151–152. Medically important fungi: a guide to identification, 5th ed ASM Press, Washington, DC. 2011.
72. Ates An, Ozcan K, Ilkit Mt. Diagnostic value of morphological, physiological and biochemical tests in distinguishing Trichophyton rubrum from Trichophyton mentagrophytes complex. Sabouraudia. 2008;46(8):811-22.
73. Hutvagner G. Small RNA asymmetry in RNAi: function in RISC assembly and gene regulation. FEBS letters. 2005;579(26):5850-7.
74. Kapranov P, Cheng J, Dike S, Nix DA, Duttagupta R, Willingham AT, et al. RNA maps reveal new RNA classes and a possible function for pervasive transcription. Science. 2007;316(5830):1484-8.
75. Perkins DO, Jeffries C, Sullivan P. Expanding the 'central dogma': the regulatory role of nonprotein coding genes and implications for the genetic liability to schizophrenia. Molecular psychiatry. 2005;10(1):69-78.
76. Yutin N, Koonin EV. Hidden evolutionary complexity of Nucleo-Cytoplasmic Large DNA viruses of eukaryotes. Virology journal. 2012;9(1):1.
77. Taft RJ, Pang KC, Mercer TR, Dinger M, Mattick JS. Non-coding RNAs: regulators of disease. The Journal of pathology. 2010;220(2):126-39.
78. Bartel DP. MicroRNAs: target recognition and regulatory functions. cell.2009;136(2): 215-33.
79. Karolchik D, Kuhn RM, Baertsch R, Barber GP, Clawson H, Diekhans M, et al. The UCSC genome browser database: 2008 update. Nucleic acids research. 2008;36(suppl 1):D773-D9.
80. Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. Cell. 2009;136(4):629-41.
81. Ørom UA, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, et al. Long noncoding RNAs with enhancer-like function in human cells. Cell. 2010;143(1):46-58.
82. Kim T-K, Hemberg M, Gray JM, Costa AM, Bear DM, Wu J, et al. Widespread transcription at neuronal activity-regulated enhancers. Nature. 2010;465(7295):182-7.
83. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proceedings of the National academy of Sciences of the United States of America. 2004;101(9):2999-3004.
84. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, et al. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. cell. 2007;129(7):1311-23.
85. Brosius J. Waste not, want not–transcript excess in multicellular eukaryotes. TRENDS in Genetics. 2005;21(5):287-8.
86. Struhl K. Transcriptional noise and the fidelity of initiation by RNA polymerase II. Nature structural & molecular biology. 2007;14(2):103-5.
87. Kanellopoulou C, Monticelli S, editors. A role for microRNAs in the development of the immune system and in the pathogenesis of cancer. Seminars in cancer biology; 2008: Elsevier.
88. Kim M, Kasinski AL, Slack FJ. MicroRNA therapeutics in preclinical cancer models. The lancet oncology. 2011;12(4):319-21.
89. Li M, Li J, Ding X, He M, Cheng S-Y. microRNA and cancer. The AAPS journal. 2010;12(3):309-17.
90. McManus MT, editor MicroRNAs and cancer. Seminars in cancer biology; 2003: Elsevier.
91. Montano M. MicroRNAs: miRRORS of health and disease. Translational research. 2011;157(4):157-62.
92. Negrini M, Nicoloso MS, Calin GA. MicroRNAs and cancer—new paradigms in molecular oncology. Current opinion in cell biology. 2009;21(3):470-9.
93. Ruan K, Fang X, Ouyang G. MicroRNAs: novel regulators in the hallmarks of human cancer. Cancer letters. 2009;285(2):116-26.
94. Lee RC, Feinbaum RL, Ambros V. TheC. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75(5):843-54.
95. Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans .Science. 2001;294(5543):858-62.
96. Coutinho LL, Matukumalli LK, Sonstegard TS, Van Tassell CP, Gasbarre LC, Capuco AV, et al. Discovery and profiling of bovine microRNAs from immune-related and embryonic tissues. Physiological genomics. 2007;29(1):35-43.
97. Büssing I, Slack FJ, Großhans H. let-7 microRNAs in development, stem cells and cancer. Trends in molecular medicine. 2008;14(9):400-9.
98. Cho WC. MicroRNAs in cancer—from research to therapy. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer .2010;1805(2):209-17.
99. Cho WC. MicroRNAs: potential biomarkers for cancer diagnosis, prognosis and targets for therapy. The international journal of biochemistry & cell biology. 2010;42(8):1273-81.

100. Giovannetti E, Erozenci A, Smit J, Danesi R, Peters GJ. Molecular mechanisms underlying the role of microRNAs (miRNAs) in anticancer drug resistance and implications for clinical practice. Critical reviews in oncology/hematology. 2012;81(2):103-22.
101. Zhao Y, Srivastava D. A developmental view of microRNA function. Trends in biochemical sciences. 2007;32(4):189-97.
102. Wouters MD, van Gent DC, Hoeijmakers JH, Pothof J. MicroRNAs, the DNA damage response and cancer. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2011;717(1):54-66.
103. Rukov JL, Shomron N. MicroRNA pharmacogenomics: post-transcriptional regulation of drug response. Trends in molecular medicine. 2011;17(8):412-23.
104. Schaefer A, Jung M, Kristiansen G, Lein M, Schrader M, Miller K, et al., editors. MicroRNAs andcancer: current state and future perspectives in urologic oncology. Urologic Oncology: Seminars and Original Investigations; 2010: Elsevier.
105. Voorhoeve PM, Agami R. Classifying microRNAs in cancer: the good, the bad and the ugly. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer. 2007;1775(2):274-82.
106. Wiemer EA. The role of microRNAs in cancer: no small matter. European journal of cancer. 2007;43(10):1529-44.
107. McDaneld T. MicroRNA: mechanism of gene regulation and application to livestock .Journal of animal science. 2009;87(14_suppl):E21-E8.
108. Babashah S, Soleimani M. The oncogenic and tumour suppressive roles of microRNAs in cancer and apoptosis. European journal of cancer. 2011;47(8):1127-37.
109. Lund E, Güttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. Science. 2004;303(5654):95-8.
110. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. nature. 2000;404(6775):293.
111. Jackson RJ, Standart N. How do microRNAs regulate gene expression? Sci Stke. 2007;2007(367):re1-re.
112. Hatakeyama H, Cheng H, Wirth P, Counsell A, Marcrom SR, Wood CB, et al. Regulation of heparin-binding EGF-like growth factor by miR-212 and acquired cetuximab-resistance in head and neck squamous cell carcinoma. PloS one. 2010;5(9):e12702
113. Huggett J, Dheda K, Bustin S ,Zumla A. Real-time RT-PCR normalisation; strategies and considerations. Genes and immunity. 2005;6(4):279.
114. Bustin S, Benes V, Nolan T, Pfaffl M. Quantitative real-time RT-PCR–a perspective. Journal of molecular endocrinology. 2005;34(3):597-601
115. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods. 2001;25(4):402-8.
116. Soheila R.A Study on the Structure and Function of Micro RNA and Its Role in Cancer. Genetics in the third millennium. 2011.
117. Hanahan D, Weinberg RA .Hallmarks of cancer: the next generation. cell. 2011;144(5):646-74.
118. Shahrokh I. Gastric cancer a multifactorial disease J Army univ med sci 2013;11(2):157-64.
119. Dong LM, Potter JD, White E, Ulrich CM, Cardon LR, Peters U. Genetic susceptibilityto cancer: the role of polymorphisms in candidate genes. Jama. 2008;299(20):2423-36.
120. Zagari RM, Bazzoli F. Gastric cancer: who is at risk? Digestive Diseases. 2004;22(4):302-5.
121. Honardoost MA, Kiani-Esfahani A, Ghaedi K, Etemadifar M, Salehi M. miR-326 and miR-26a, two potential markers for diagnosis of relapse and remission phases in patient with relapsing–remitting multiple sclerosis. Gene. 2014;544(2):128-33.
122. Hauser SL, Oksenberg JR. The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration. Neuron. 2006;52(1):61-76.
123. Goodin DS. Magnetic resonance imaging as a surrogate outcome measure of disability in multiple sclerosis: have we been overly harsh in our assessment? Annals of neurology. 2006;59(4):597-605.
124. Rudick RA, Lee JC, Simon J, Fisher E. Significance of T2 lesions in multiple sclerosis: A 13-year longitudinal study. Annals of neurology. 2006;60(2):236-42.
125. Honardoost MA, Mesrian Tanha H, Etemadifar M, Rahgozar S, Salehi M, Ghaedi K. The Role ofmiRNAs in Multiple sclerosis. Genetics in the third millennium .2014.
126. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, et al. Requirement of bic/microRNA-155 for normal immune function. Science. 2007;316(5824):608-11.
127. Vigorito E, Perks KL, Abreu-Goodger C, Bunting S, Xiang Z, Kohlhaas S, et al. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. Immunity. 2007;27(6):847-59.
128. Thai T-H, Calado DP, Casola S, Ansel KM, Xiao C, Xue Y, et al. Regulation of the germinal center response by microRNA-155. Science. 2007;316(5824):604-8.
129. Haasch D, Chen Y-W, Reilly RM, Chiou XG, Koterski S, Smith ML, et al. T cell activation induces a noncoding RNA transcript sensitive to inhibition by immunosuppressant drugs andencoded by the proto-oncogene, BIC. Cellular immunology. 2002;217(1):78-86.
130. Faraoni I, Antonetti FR, Cardone J, Bonmassar E. miR-155 gene: a typical multifunctional microRNA. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease. 2009;1792(6):497-505.
131. Junker A, Krumbholz M, Eisele S, Mohan H, Augstein F, Bittner R, et al. MicroRNA profiling of multiple sclerosis lesions identifies modulators of the regulatory protein CD47. Brain. 2009;132(12):3342-52.
132. Du C, Liu C, Kang J, Zhao G, Ye Z, Huang S, et al. MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. Nature immunology. 2009;10(12):1252-9.
133. Mina N. The study of changes in the expression of miR-222 in gastric cancer associated with Helicobacter pylori. 1393.

134. Hajar MzM. Genetic basis of metabolic syndrome. Jornal of Isfehan Medical School(JIMS). 1394:355-67.
135. Barker KR, Lu Z, Kim H, Zheng Y, Chen J, Conroy AL, et al. miR-155 modifies inflammation, endothelial activation and blood-brain barrier dysfunction in cerebral malaria. Molecular Medicine. 2017;23:24.
136. Hong Y, Fu Z, Cao X, Lin J. Changes in microRNA expression in response to Schistosoma japonicum infection. Parasite Immunology. 2017;39(2).
137. Yang X ,Li H, Sun H, Fan H, Hu Y, Liu M, et al. Hepatitis B Virus-Encoded MicroRNA Controls Viral Replication. Journal of Virology. 2017;91(10):e01919-16.
138. Casselli T, Qureshi H, Peterson E, Perley D, Blake E, Jokinen B, et al. MicroRNA and mRNA Transcriptome Profiling in Primary Human Astrocytes Infected with Borrelia burgdorferi. PloS one. 2017;12(1):e0170961.
139. Lu P, Fang C, Cheng Q, Ke WJ, Huang T, Zhang J, et al. Serum microRNA profiles in patients with syphilis. Journal of the European Academy of Dermatology and Venereology. 2017.
140. He J-J, Ma J, Wang J-L, Xu M-J, Zhu X-Q. Analysis of miRNA expression profiling in mouse spleen affected by acute Toxoplasma gondii infection. Infection, Genetics and Evolution. 2016;37:137-42.
141. Amir Y. Medical Mycology introduction to Medical Mycology Superficial Mycoses and Saprophytic Fungi. Iran University Of Medical Sciences: 1; 1386. 36-44 p.
142. Youssef N, Wyborn C, Holt G, Noble W, Clayton YM. Antibiotic production by dermatophyte fungi. Microbiology. 1978;105(1):105-11.
143. Nishimura T, Nakamura K, Yamashita S, Ikeda S, Kigure K, Minegishi T. Effect of the molecular targeted drug, erlotinib, against endometrial cancer expressing high levels of epidermal growth factor receptor. BMC cancer. 2015;15(1):957.
144. Mohsenzadegan M, Fayazi MR, Abdolmaleki M, Bakhshayesh M, Seif F, Mousavizadeh K. Direct immunomodulatory influence of IFN-β on human astrocytoma cells. Immunopharmacology and immunotoxicology. 2015;37(2):214-9.
145. Yuan JS, Reed A, Chen F, Stewart CN. Statistical analysis of real-time PCR data. BMC bioinformatics. 2006;7(1):85.
146. Grandis JR, Melhem MF, Gooding WE, Day R, Holst VA, Wagener MM, et al. Levels of TGF-α and EGFR protein in head and neck squamous cell carcinoma and patient survival. JNCI: Journal of the National Cancer Institute. 1998;90(11):824-32.
147. Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. New England Journal of Medicine. 2006;354(6):567-78.
148. Vermorken JB, Mesia R, Rivera F, Remenar E ,Kawecki A, Rottey S, et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. New England Journal of Medicine. 2008;359(11):1116-27.
149. Khambata-Ford S, Garrett CR, Meropol NJ, Basik M, Harbison CT, Wu S, et al. Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. Journal of clinical oncology. 2007;25(22):3230-7.
150. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. science. 2007;316(5827):1039-43.
151. Cohen EE, Lingen MW, Martin LE, Harris PL, Brannigan BW, Haserlat SM, et al. Response of some head and neck cancers to epidermal growth factor receptor tyrosine kinase inhibitors may be linked to mutation of ERBB2 rather than EGFR. Clinical cancer research. 2005;11(22):8105-8.
152. Tran N, McLean T, Zhang X, Zhao CJ, Thomson JM, O'Brien C, et al. MicroRNA expressionprofiles in head and neck cancer cell lines. Biochemical and biophysical research communications. 2007;358(1):12-7.
153. Simanski M, Rademacher F, Schröder L, Schumacher HM, Gläser R, Harder J. IL-17A and IFN-γ synergistically induce RNase 7 expression via STAT3 in primary keratinocytes. PloS one. 2013;8(3):e59531.