1. Project Title: Post-transcriptional regulation of biological drugs response in patients with rheumatoid arthritis

2. Introduction

MicroRNAs (miRNAs) are small (~22 nucleotides), non-protein coding RNA molecules known to regulate the expression of genes by binding to the 3’-untranslated regions (3’-UTR) of mRNAs. They are involved in the negative post-transcriptional regulation of gene expression. Their inhibitory action is exerted by binding to the 3’-UTR region of nascent mRNA transcripts together with several other helper proteins, and in mammals it is observed mainly as an inhibition of protein synthesis. These non-protein coding RNA molecules are master molecular regulators that have been found to be involved in cellular processes ranging from differentiation, cell division, signal transduction and apoptosis. Furthermore, the transition from a healthy to diseased tissue occurs in association with remarkable changes in the expression of miRNAs, either as direct cause of the disease or as a side effect of other regulatory modifications in cell.

Pharmacogenomics is the study of the influence of patient’s genome differences in the effectiveness and safety of drugs. Given the inherent difference between patients, pharmacogenomics aims to determine the best personalized medical treatment, thereby maximizing the response to the medication and minimizing its adverse effects. These studies have important implications not only in drugs development and safety, but also in clinical practice.

Immune-mediated rheumatic diseases (IMRD) are complex diseases starting at young and middle-ages and affecting individuals during their entire life. Chronic inflammation and structural damage are the common denominator among these disabling entities. In the last decade, biotechnological agents emerged as one of the most promising therapies in immune-mediated rheumatic diseases. Anti-tumour necrosis factor (TNF) agents are the first line approved biological therapies. Nevertheless, more than 30% of the patients remain refractory to these new treatments or develop toxicity which leads to therapy discontinuation. Short and long term biological drugs toxicities represent a major concern. Hypersensitivity reactions can be life-threatening. Viral, bacterial and opportunistic infections can occur and sometimes are manifested by atypical clinical features, can be recurrent and refractory to standard therapies. Neoplasm recurrence, increased risk of lymphomas, demyelinating disease, pancytopenia, autoimmune manifestations, cardiovascular complications and fulminant hepatitis were reported in association with biological therapies namely to anti-TNF agents.
The relationship between miRNAs and therapeutic response is indirectly established through the genes they regulate [1]. Moreover, there is direct evidence of connectivity between the immune response of mammals and miRNAs [2-9], and growing evidence also reveals the differential expression of certain immunity-regulating miRNA in rheumatoid patients [10-18]. The effectiveness of many drugs depends on the correct expression of specific genes [19]. The overexpression of specific miRNAs could decrease the expression levels of genes that promote drug's effectiveness. Conversely, if some miRNAs are under expressed may increase the expression levels of genes that inhibit the effectiveness of the drug treatment.

It is estimated that miRNAs regulate the expression of more than half of human genes [20]. Due to the key role of miRNAs in the regulation of gene expression, it is not surprising that many important genes in drug response are also targets of miRNAs, stressing the need of taking miRNAs into consideration when conducting pharmacogenomic studies [20-23]. The regulation by miRNAs of the expression of genes related to the effectiveness of a given drug is the basis of what is called “miRNA pharmacogenomics” and which underlies the “personalized medicine” [24][25].

2.1. Previous Studies

Early studies on the role of miRNAs in pharmacogenomics investigated SNPs in regions of mRNA that are miRNA targets [26-28]. The basic principle consists in showing that polymorphisms in target mRNA sequences of miRNAs can affect the expression levels of genes relevant pharmacogenomics, affecting the efficacy of the drugs. However, until the present date, it has only been reported downstream consequences for the function of methotrexate due to an SNP in the target sequence corresponding to miR-24 DHFR gene, an important protein in the folic acid cycle [29].

Studies on miRNAs tell us that, rather than differences in sequences (such as SNPs), differences in expression of miRNAs in specific tissues or between individuals are crucial for the impact of miRNAs in genes regulation. In fact, the number of pharmacogenomic sets (composed of miRNAs, genes and drugs) described in literature has increased. However, most of these studies did not investigate SNPs, but the differences in expression levels of miRNAs that affect the drugs response [30-34].

Most of the knowledge regarding the role of miRNAs in pharmacogenomics is currently associated with the response to chemotherapeutic treatments. This tendency can be partly explained not only by the impact of cancer diseases in today's society, as well by the fact that it is relatively easy the detection of treatment response phenotypes (typically proliferation or apoptosis) in human cancer cell lines. To date, few pharmacogenomics investigations through miRNAs and related with rheumatic diseases are known.
Another one of the limitations of current research is related to the fact that are invariably used models involving a single miRNA, a single gene and a drug. This model may not take into account all the regulatory mechanism of miRNAs because, for example, several targets of the same miRNA may be involved in the response to therapy. Similarly, drug resistance may result from the combined actions of various miRNAs because there are often multiple deregulated miRNAs in tissues affected by the disease. Finally, the deregulation of a miRNA might just be an early event in a signaling cascade involving several genes. As previously mentioned, these regulatory networks are not fully understood and identified. As such, further studies are also needed in this area.

One last thing to mention about the research related to miRNAs lies in the fact that it is relatively recent. Actually the first miRNAs were discovered in 1993, and during the following 10 years, all discovered miRNAs were intergenic (located in non-coding regions between genes). Only in 2003, intronic miRNAs, i.e. produced from introns were discovered. Consequently, all gained knowledge has been constantly evolving, resulting in new versions of the various databases used in the investigations that are being conducted. For this reason, repeating experiments already performed, using the new versions of databases, is often justified. Nevertheless, we are unaware of any other investigations related to the role of miRNAs in the response to biotechnological drugs for patients with rheumatoid arthritis.

2.2. Hypothesis and Motivation for this Research

Our research hypothesis assumes, like other studies, that the analysis of expression levels of miRNAs is potentially more promising for studies in the field of pharmacogenomics than the detection of polymorphisms in genes targeted by miRNAs. On one hand, the number of different miRNAs is much lower than the variety of genes, causing its analysis to be simpler than the analysis of the universe of genes. Moreover, as noted above, miRNAs are master regulators and molecular differences in their expression level affect many genes. That is, disturbances in expression levels of a single miRNA can have significant impact on various genes, changing the drug response.

Assuming our investigation hypothesis, our primary aim will be to detect variations in the level of expression of miRNAs that may be associated with therapeutic response and / or immune-mediated adverse reactions in patients with rheumatoid arthritis treated with biological agents (anti-TNF). As noted above, other similar investigations in the field of rheumatology are rare, and given the impact of these diseases in the general population as well as the high cost of treatments, it is pertinent that these investigations are carried out.

Moreover, this study intends to use more complex models than those traditionally used, identifying regulatory networks and signalling cascades simultaneously involving several miRNAs and different genes. To achieve this, we will use the most recent versions of various databases.
databases predicting the genes that bind to the promoter sequences of miRNAs, and several databases predicting the targets of miRNAs.

3. Objectives

The first objective of this study is to investigate differences in expression of miRNAs in groups of patients with rheumatoid arthritis treated with biological therapeutics (TNF blocking agents). These individuals will be previously classified according to the response to the therapy, both in terms of disease activity and the adverse events associated with the drug. The second objective of this work is to identify how the differences in the expression of miRNAs may be associated with the response to therapy. These objectives, if successfully achieved, will allow us to identify miRNAs and genes that modulate responses to drugs, affect the pharmacokinetics and pharmacodynamics of drugs and/or are associated with adverse reactions. To achieve the second objective of this study is essential to take into consideration that, as all the transcriptional units, the genesis of miRNAs is dependent of transcription factors. Moreover, most of the target sequences in the 3'-UTR region of the mRNA only have partial bases complementarily with the corresponding miRNAs. As such, a single miRNA is regulated and can regulate the expression of numerous genes. Furthermore, a mRNA can contain multiple binding sequences for different miRNAs, hence resulting complex regulatory networks. Since a single miRNA can regulate multiple genes and a single gene can be regulated by multiple miRNAs, it seems logical to think that miRNAs and transcription factors may cooperate in the regulation of target genes at both the transcriptional and post-transcriptional levels. In fact, transcription factors and miRNAs work together in regulatory networks of genes that are not yet fully identified or understood.

4. Methods

This study will have a pilot phase involving the selection and assessment of 10 patients according to response to therapy, both in terms of disease activity and the adverse events associated with the drug. For this pilot study and regarding the drug response, patients with at least 2 years in biological therapy, without switching treatment and having DAS28 values less than 3.2 (disease in remission or low activity) for at least 18 consecutive months, were considered good responders. Patients selected as poor responders also have at least 2 years in biological therapy, but made one or more treatment switches, and always or almost always had values of DAS28 over 3.2 (moderate or high disease activity). To avoid misinterpretation in any
ambiguous situations six individuals without rheumatic diseases diagnosed will be also analyzed. This pilot study will allow us to formulate more specific research objectives or setting goals beyond those already planned, depending on the obtained results.

We anticipate that a total of 60 adults diagnosed with rheumatoid arthritis and treated with biological drugs will be included in this research project, excluding pregnant or breastfeeding. The control group will consist of 30 individuals considered healthy. All patients already have blood sample stored in the IMM Biobank with their informed consent and also have the clinical information registered in the Rheumatic Diseases Portuguese Register (Reuma.pt), both approved by the CNDP and the ethics committee of the HSM. For screening the expression of miRNAs will be used low density arrays based on quantitative PCR and marketed by Exiqon, using as a basis the RNA extracted from the serum of blood samples from selected patients. The quantification of the expression levels of miRNAs will be performed by quantitative PCR using primers of LNAs (lock nucleic acids) also marketed by Exiqon.

To achieve the second objective, i.e., to understand how any differences in the expression of miRNAs may be associated with response to therapy, will use databases such the PharmGKB - Pharmacogenomics Knowledge Base [40] and Pharmaco miR - The miRNA Pharmacogenomics Database [41], public databases predicting the genes that bind to the promoter sequences of miRNAs [42-47] and several databases predicting the targets of miRNAs [48-54]. It is fundamental to cross the information from these various databases because many authors have postulated the existence of regulation loops between transcription factors that control the expression of miRNAs and the regulation exerted by miRNA over the expression of transcription factors.

The statistical analysis of miRNA expression will include the calculation of Fold Regulation, Fold Change with p-values and 95% confidence interval, average Ct (threshold cycle) values, ΔCt value between GOI and HKG for each experiment, average ΔCt values between experiments and ΔCt values (ΔCt experiment - ΔCt control). Volcano plots scatter plots, clustergrams and 3-D histograms will also be analyzed. In addition to descriptive and inferential statistics, will use machine learning techniques and algorithms that will allow us to analyze the data and extract useful knowledge from them. The machine learning is a field of research that encompasses knowledge from several areas, including statistics, artificial intelligence, databases and information systems.

5. Expected results

From the first laboratorial component of the study it is expected to obtain a set of miRNAs differentially expressed in the two three of analyzed patients (good and bad responders, and
healthy subjects). Achieving these results will be crucial in order to be able to proceed with the study and seek to achieve the following objectives.

The expected outcome from analysis of the miRNAs-drug response associations is the characterization of regulation loops involving miRNAs and genes, and to identify how these interactions can influence the response to therapy and/or how they may be associated with adverse events.

The last expected outcome is the portal rheuma-mir.org that will provide algorithms and data resulting from all previous work, allowing future investigations.

6. Timeline and Milestones

- December 2013 - screening of miRNA expression for the pilot study samples
- January 2014 to September 2014 - Analysis of data from the pilot study and publication of an original article
- October 2014 to September 2015 - screening of miRNA expression of the remaining patients and controls, the analysis of all data and to publish at least one original article
- October 2015 to September 2016 - the identification of regulatory networks involving miRNAs and transcription factors potentially associated with response to therapy, as well as publication of at least one original article
- October 2016 to September 2017 - design and development of the portal rheuma-mir.org, in order to provide algorithms and data resulting from the previous work, allowing future investigations; as well to publish an original article describing this tool

7. Research Team, Institutions and Authorship

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7.6. Authorship: Each centre may indicate a contributor by each 3 patients used in this study. The authorship and co-authoring of the published work will be governed by the "Rules of Vancouver". Contributors who do not meet the criteria for authorship will be listed in the acknowledgments section.

8. Funding and Conflicts of Interest

None.

9. Bibliography


