Gene 647 (2018) 157–163

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Systems biomarkers in psoriasis: Integrative evaluation of computational and experimental data at transcript and protein levels

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ARTICLE INFO

Keywords:
Autoimmune disease
Gene clusters
Psoriasis
Systems biomarkers

ABSTRACT

Psoriasis is a complex autoimmune disease with multiple genes and proteins being involved in its pathogenesis. Despite the efforts performed to understand mechanisms of psoriasis pathogenesis and to identify diagnostic and prognostic targets, disease-specific and effective biomarkers were still not available. This study is compiled regarding clinical validation of computationally proposed biomarkers at gene and protein expression levels through qRT-PCR and ELISA techniques using skin biopsies and blood plasma. We identified several gene and protein clusters as systems biomarkers and presented the importance of gender difference in psoriasis. A gene cluster comprising of *PI3*, *IRF9*, *IFIT1* and *NMI* were found as positively correlated and differentially co-expressed for women, whereas *SUB1* gene was also included in this cluster for men. The differential expressions of *IRF9* and *NMI* in women and *SUB1* in men were validated at gene expression level via qRT-PCR. At protein level, PI3 was abundance in disease states of both genders, whereas PC4 protein and WIF1 protein were significantly higher in healthy states than disease states of male group and female group, respectively. Regarding abundancy of PI3 and WIF1 proteins in women, and PI3 and PC4 in men may be assumed as systems biomarkers at protein level.

1. Introduction

Psoriasis is an immune mediated disease affecting the skin and joints. Previous studies showed that there is a complex interplay between the innate and adaptive immune system during disease progression in response to an unidentified trigger which can be either genetic, environmental or immunologic (Perera et al., 2012). Its prevalence rate across the globe varies between 0.09% and 11.43% (World Health Organization, 2016). To date there is no cure for psoriasis and its treatment only results in a temporary remission of physical symptoms (Schleicher, 2016). Patients have to endure the disease daily which has an adverse effect on their quality of life. Thus, it is of importance to discover prognostic molecular signatures of the disease which will be useful in the development of effective treatment strategies.

Over the last decades, enormous research has been done to understand mechanisms of psoriasis pathogenesis and to identify diagnostic and prognostic targets (Trembath et al., 1997; Nair et al., 2000; Oestreicher et al., 2001; Koczan et al., 2005; Sa et al., 2007; Johnston et al., 2013; D'Erme et al., 2015). The existence of serious comorbidities such as cardiovascular disease, metabolic syndrome and diabetes (Gottlieb et al., 2008; Karadag et al., 2010; Karadag et al., 2013) brings additional gravity to biomarker discovery in psoriasis. Despite these efforts, disease-specific and effective biomarkers were still not available, since studies have focused on individual genes or proteins, ignoring the interactions and associations among the gene products.

Technological advances are directing research efforts towards the integration of omics platforms in the expectation of discovering efficient biomarkers and establishing cures for complex diseases such as cancers, diabetes and psoriasis (Boja et al., 2014; Karagoz et al., 2015; Calimlioglu et al., 2015; Sinha et al., 2016; Ayyildiz et al., 2017; Dayan et al., 2017; Gov and Arga, 2017). With complex diseases such as psoriasis, integration efforts are not coming forth quick enough. There

Abbreviations: 4PL, 4 parameters logistic regression; DEGs, Differentially expressed genes; ELISA, Enzyme-linked immunosorbent assays; FC, Fold change; GEO, Gene Expression Omnibus; LC/GC–MS, Liquid chromatography/Gas chromatography–mass spectrometry; PASI, Psoriasis Area Severity Index; PCC, Pearson correlation coefficients; PCR, Polymerase chain reaction; qRT-PCR, Real-time quantitative reverse transcription PCR; SD, Standard deviation

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https://doi.org/10.1016/j.gene.2018.01.033
Received 19 October 2017; Received in revised form 6 December 2017; Accepted 8 January 2018
Available online 09 January 2018
0378-1119/

is still a lack of a cure and the disease mechanism is still under question.

In our previous study, in order to increase dimensionality and breadth of information that can be extracted, we performed a meta-analysis of transcriptome data from twelve independent studies reported in literature, and within a computational systems biology framework, topological, and modular analyses of gene co-expression, protein–protein interaction, and transcriptional regulatory networks (Sevimoglu and Arga, 2015). In hopes of illuminating psoriasis disease components this experimental study is a follow up of a previous integromics effort done in genomics, transcriptomics and proteomics levels. The analysis of the transcriptomic data in conjunction with proteomic data may present valuable understanding that might not have been possible by the investigation of mRNA or protein expressions alone. Therefore, in the present study, potential biomarkers of psoriasis were analyzed at gene and protein expression levels, experimentally and discussed under the perspective of gender effect on disease. The results of transcriptome meta-analysis were validated through real-time quantitative reverse transcription PCR (qRT-PCR) technique using skin biopsies. Moreover, enzyme-linked immunosorbent assays (ELISA) were also conducted to examine differential expression of potential biomarkers at protein level, since it is more efficient to look for proteomics biomarkers using blood and plasma of patients in clinical trials.

2. Materials and methods

2.1. Selection of candidate biomarkers

Previously, transcriptome data from twelve datasets considering total of 534 samples were extracted from the Gene Expression Omnibus (GEO) and integrated with biomolecular networks to identify potential biomarkers and therapeutic targets (Sevimoglu and Arga, 2015). As results of the computational evaluation of psoriasis gene expression data, JAK/STAT signaling pathway involving cytokines, interferon-stimulated genes, anti-microbial peptides were proposed as significant, and several biomarker and therapeutic gene/protein candidates were proposed for future experimental studies. Here, among those candidates, *IFI44*, *SUB1*, *IFIT1*, *OAS2*, *PI3*, *RSAD2*, *NMI*, *WIF1* and *IRF9* were selected for evaluation of gene expressions, whereas elafin protein expressed by *PI3* gene, PC4 protein expressed by *SUB1* gene and WIF1 protein were chosen to be pursued at protein level since these proteins are secreted extracellular and hence easy to chase as a biomarker from patients' blood (Fig. 1).

2.2. Co-expression analysis among candidate systems biomarkers considering gender differences

Datasets comprising information on gender differences, GSE34248 (Bigler et al., 2013), GSE6710 (Reischl et al., 2007) and GSE42632 (Niu and Zhang, 2016), were downloaded from GEO. Expression profiles within diseased and healthy states were extracted to construct two new subsets for men and women consisting total of 49 and 17 samples, respectively. Each dataset was normalized by Robust Multi-Array method to reduce batch effects and ensure a similar empirical distribution of each array. Pearson correlation coefficients (PCC) were calculated between every pair of candidate marker in each gender. PCC cut-offs of ≥ 0.70 (positive correlation) and ≤ 0.70 (negative correlation) were employed to determine statistical significance of the pairwise co-expressions. Consequently, two co-expression networks representing diseased and healthy states were established for both genders, and differential co-expression (Gov and Arga, 2017) patterns were analyzed.

2.3. Patients' characteristics

The study was conducted under the approval by the ethical committee of the Istanbul Medeniyet University. The patients received a written description of the sampling procedure and study purpose, as well as the planned use and storage of the information they were to provide. This was followed by a description of the subject's rights according to the Helsinki Declaration. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 and 2008. Written informed consent was obtained from all patients for use of their medical records being included in the study. After patient consent, blood and skin samples were taken from 4 female and 3 male psoriasis patients and 3 female and 2 male healthy controls with ages ranging between 20 and 48. The patients' Psoriasis Area and Severity Index (PASI) scores, which measure the severity and extent of psoriasis, were ranging between 5 and 13.

2.4. RNA isolation

Skin samples were taken using 4 mm punch biopsies. The samples were flash-frozen in liquid nitrogen and stocked at −80 °C until RNA isolation. Cell disruption was carried out using Cellcrusher®. Total RNA isolation was performed using the RNeasy® Mini Kit (Cat.No. 47104-Qiagen, Milano, Italy) according to manufacturer's protocols. The RNA concentration and purity were determined spectrophotometrically using IMPLEN® Nanophotometer P-Class. Subsequently, all the RNA samples were diluted to the same concentration for further PCR analyses.

2.5. Evaluation of candidate psoriasis biomarkers using qRT-PCR analysis

Primers were designed using Primer3Plus (Untergasser et al., 2012) and PrimerQuest software except for *OAS2* (Schmeisser et al., 2010) (Table 1). The *RPLP0* gene encoding the 60S ribosomal protein was used as the housekeeping gene. qRT-PCR was carried out on the LightCycler using Lightcycler® RNA Master SYBR Green I kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. The 20 µl reaction mixture consisted of 7.5 µl RNA master SYBR Green I, 1.3 µl $MN(OAc)_2$ stock solution, 0.5 µl (from 10 µM stock primer solution) of each primer, 1 µl of diluted RNA and RNase-free water to complete the 20 µl volume.

The qRT-PCR program was as follows: 20 m at 61 °C for reverse transcription step, 30 s at 95 °C for initial denaturation step, 45 cycles for amplification step consisting of 5 s at 95 °C, 10 s at (primer-dependent) °C and 30 s at 72 °C for denaturation, annealing, and extension respectively, and finally 1 cycle for melting curve step consisting of 0 s at 95 °C, 15 s at 65 °C, and 0 s at 95 °C for denaturation, annealing, and melting respectively. All the PCR runs were performed in triplicate and included a negative control reaction using RNase-free water instead of template. The averages of the results were used for further evaluations. The fold changes (FC) were calculated according to the ΔΔCt comparative quantification method as described by Livak and Schmittgen (2001).

2.6. Screening of biomarker candidates at protein level via ELISA

Three extracellular proteins from nine candidates were chosen for screening protein biomarkers of psoriasis. The plasma was collected using heparin as an anticoagulant and centrifuged for 15 min at $1000 \times g$ at 2–8 °C within 30 min of collection. Samples were aliquoted and stored at −80 °C. Commercial ELISA kits for elafin protein expressed by PI3 gene (Abnova, Taiwan), PC4 protein expressed by SUB1 gene and WIF1 protein (BlueGene, China) were used according to the manufacturer's protocols to identify the pre-selected protein concentrations in plasma of patients and healthy controls. The absorbance at 450 nm was quantitated by an 8-channel spectrophotometer (EL800 Universal Microplate Reader; BIO-TEK Instruments Inc.). Serial dilutions of samples were tested to find the best dilution rate in the absorbance range.