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Molecular Evaluation of Heat Shock Protein 90 in Oral Lichen Planus, A Reassuring Marker for Targeted Therapy in Oral Lichen Planus: A New Perspective in the Treatment of Oral Lichen Planus

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Abstract

Background: Oral lichen planus (OLP) is considered as a chronic inflammatory mucocutaneous disorder. It has also been classified as a potentially malignant component in the oral counterpart lesion (OLP) The treatment modality is based on the clinical presentation of the lesion. The current regimen follows to control the disease only since there is no full effective treatment found yet. The treatment of choice involves topical or systemic corticosteroids, but other drugs may also be used. Heat shock proteins (HSPs) are dynamized by both Environmental and patho-physiologic stresses thus enabling the cells to survive from stressful conditions. Heat shock proteins show an increased expression in a range of human cancers. Yet the role of HSP' s in carcinogenesis is ambiguous. The aim of this paper helps us identify the Heat shock protein 90 as a potential and promising target in the treatment and management.

Methodology: Real time PCR was performed for 90 samples. The RNA was extracted using TRIZOL. cDNA was reverse-transcribed. HSP90 primers and beta actin primers. HSP90 and beta-actin in parallel with were amplified in triplicate.

Results: Showed increase in expression from normal when compared to study groups. This was measured by fold changes. As the disease progressed, levels of HSP 90 gene expression also increased accordingly and was found statistically significant ($p<0.0001$) amongst the study groups.

Conclusion: The role of HSP 90 has been identified by genomic analysis. This forms a clear foundation for further research to develop targeted therapy for lichen planus targeting HSP 90 gene.

keywords: Oral lichen planus; Heat shock protein 90; Targeted therapy

Introduction

Oral lichen planus (OLP) is considered as a chronic inflammatory disease and a potentially malignant disorder with a malignant transformation rate of 0.5-2% [1]. The commonly involved sites are buccal mucosa, gingiva and tongue. It clinically presents as bilateral white striations or papules on the involved sites. Erosions and blistering along with erythema may be seen. Almost 50% of patients who have oral lichen planus also have skin lesions. Skin lesions in LP usually resolve within 1-2 years where as in OLP they persist if 20 years or more. OLP affects approximately 1-2 % of the general adult population, all races can be affected and the female to male ratio is 1.4:1.3, [2]. As of date, there is no curative therapy. Trials with many placebo and different therapeutic modalities have been examined for treatment of lichen planus. Topical corticosteroids promote healing of erosive areas, but do not eliminate lichen planus. Systemic steroid therapy is used to reduce the submucosal

lymphatic infiltrate and inflammatory signs but at the same time adverse and long-term side effects associated with steroids such as candida overgrowth or even adrenocortical suppression restricts its usage to minimum [3].

Heat shock proteins 90 and Oral Lichen planus

Heat Shock protein 90 (HSP 90) is an essential ubiquitous protein. It is been expressed throughout the eukaryotic lineage. The protein manifests at 2 isoforms namely HSP 90 alpha (HSP 90α) and HSP 90 Beta (HSP 90β) [4]. High stress and anxiety levels have been strongly correlated as the etiological cause of OLP. Although this association has been known for decades, difficulties in objectively measuring these variables has been a limitation. Recently the importance of anxiety and stress been widely recognized; these factors are now the target of numerous studies. Soto-Araya et al. (2004) investigated the relation between psychological stress and disease of the oral mucosa which proved to be statistically significant [5]. Molecular Studies eliciting the exact role of HSP 90 in the pathogenesis of the disease and as a target for treatment for Oral lichen planus are very limited.

Pathogenesis of Oral Lichen Planus

OLP is an autoimmune disorder mediated by CD8+ T cells. They lead to auto-cytotoxic apoptosis of the basal cells of the oral squamous epithelium [6]. The basal keratinocytes thereby express or unmask an antigen which may be a self-peptide or a heat shock protein [7-9]. Subsequently, the T cells (for the most part CD8+, and some CD4+ cells) enter the squamous epithelium of the mucous membrane. This happens either due to routine surveillance to encounter an auto antigen or due to a chemokine mediated migration towards the basal keratinocyte.5 Activation of T cells takes place when the antigen (on the basal keratinocytes) binds to major histocompatabitily complex-1 (MHC-1) or through CD4+ helper cells. In addition, as the MHC-II expression gets upregulated, the Langerhan cells in OLP also show an increase in the number. This increase is followed by antigen presentation to CD4+ cells. Interleukin -12 activates CD4 + T helper cells which activate CD8+ T cells through receptor interaction, interferon γ (INF – γ) and IL-2. The activated CD8+ T cells in turn kill the basal keratinocytes through tumor necrosis factor (TNF)-α, Fas– FasL mediated or granzyme B activated apoptosis [10].

Treatment Regimen Currently followed for Oral Lichen Planus

Topical steroids have been found to be effective in treating symptomatic oral lichen planus by reducing pain and inflammation. But controversially the over usage of systemic corticosteroids in the treatment of oral lichen planus can cause adverse long-term effects in patients. Thus, the timely follow up is very essential in the Oral lichen planus patients to avoid the long-term use of steroids [11,12]. Currently the other modalities like calcineurin inhibitors, retinoids, dapsone, hydroxychloroquine, mycophenolate mofetil and enoxaparin have contributed significantly toward treatment of the disease [10]. Though the drugs have a role in treatment, none of them offer a cure for the disease. The lacuane in the treatment of OLP is targeted therapy using the self-peptide or HSP. Thus, our studies aim to provide foundation for this by experimenting the molecular mechanism of HSP 90 in the Oral lichen planus.

Aim and Objective of the Study

a. To evaluate the expression pattern of HSP 90 in OLP patients and to compare with normal, controls and those with inflamed mucosa.

b. To quantify the molecular level of HSP 90 gene in the Study groups (Normal, Inflamed and Oral lichen Planus) by real time PCR.

Study Population

Study samples were collected from patients attending outpatient department of Dermatology, Oral medicine and Oral surgery. The research proposal was approved by the Institution of Ethics, SRU in April 2012 (NI/11/OCT/25/64). A written informed consent was obtained from all the patients prior to the procedure. A total of 150 samples were collected from Department of oral medicine, Department of Oral Surgery and Department of Dermatology, Sri Ramachandra University. The slides were reviewed by the research panel. Cases with histopathology of lichenoid reactions and patients aged less than 15 years were removed from the study groups. Patients with incomplete case records were also excluded.

Materials and Methodology

Tissue Preparation

Following an institutionally approved protocol, informed consent was obtained from patients undergoing incisional biopsy at our hospital. Tissues from oral lichen planus patients with and without skin lichen planus were included in sample collection. The normal tissue samples were collected from patients who visited the institution for therapeutic removal of impacted teeth. All tissues obtained were reviewed by an oral pathologist and confirmatory diagnosis were made. Immediately after biopsy, tissue samples were frozen in liquid nitrogen and then stored at -80 °C until RNA was being extracted.

RNA Isolation

The stored samples were thawed and dissolved in Trizol from Thermo Fisher. Tissue homogenization was done. A portion of the dissolved tissue was transferred to another vial and RNA conversion was done according to protocol (Figure 1). Samples were dissolved in RNasefree water and quantified using Nanodrop. Purity of total RNA was determined by the A260/A280 and A260/A230 ratio, respectively.

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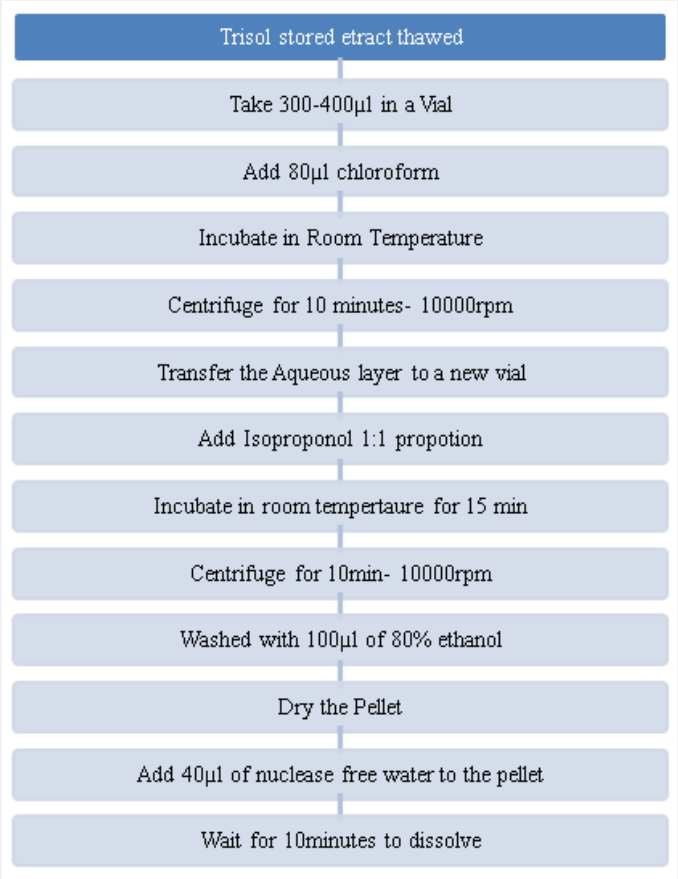

Figure 1: Steps in RNA Isolation from the tissue samples acquired.

Single Stranded cDNA Synthesis

Table1: cDNA conversion calculation shown for one sample. RNA concentration for each sample will vary according to the Nucleic acid content of the sample.

Components	Quantity (µl)
5X Prime Script Buffer	2
Prime Script RT Enzyme Mix	0.5
Oligo dT Primer	0.5
Random 6 mers	0.5
RNA (500ng)	0.712
RNase free Water	0.5.788
Total Kit	10

The cDNA synthesis was carried out using Prime Script RT Reagent Kit (TAKARA BIO, INC, INDIA). The protocol for the cDNA synthesis was followed as per the user manual. The calculations of the same are mentioned in the table below (Table 1). Each reaction was set at 10µl of which 3.5µl was used from takara kit and 6.5µl was made of RNA (500µg) and diluted with RNase free. RNA concentration was calculated based on the nucleic acid concentration obtained during the Nanodrop check. Every sample is expected to have a different RNA quantity based on the nucleic acid concentration. The remaining quantity was diluted to 10µl using RNase free water. The reaction mixture was incubated under the following conditions: 37 °C, 15 minutes (Reverse Transcription); 85 °C, 5 sec (inactivation of reverse transcriptase with heat treatment); 4 °C.

Levels of HSP90 expression were determined by real-time PCR (RT-PCR). For real-time PCR, hsp90 primers and beta actin primers were used. These primers were blasted by primer-blast site on NCBI website. The forward (F) and reverse (R) primer sequences of hsp90 and β-actin used in real-time PCR were shown in Table 2. Beta actin was used as the internal control gene. For hsp90, a 66bp amplicon and for beta actin a 648bp amplicon were generated in a 10µl reaction mixture that contained the reagents as showed (Table 3). Each RNA sample was divided into equal amounts and then, HSP90 and beta-actin in parallel with each other were amplified by real-time PCR in triplicate. Negative controls were prepared each time with 2µl DdH2O instead of the cDNA template. Real time PCR amplification was performed using an ABI system with the following setting as manufacture protocol. The reaction mixture was incubated under the following conditions: 95 °C, 2 minutes, 1 cycle (Holding step); 65 °C, 20 seconds, 45 cycles (Annealing); 72°C, 20 seconds, 45 cycles (Extension); 75-99 °C, 1 cycle (Melting)

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Table 2: Forward (F) and reverse (R) Primer Sequences of β-Actin and HSP 90 Used in Real- Time PCR.

Gene	Oligo Name	Primer Sequence	Product size
HSP 90	forHSP90F	5'-ATTGCCCAGTTGATGTCATTGA-3'	**66 bp**
	ForHSP90R	5'-ATGCATCTGATGAATTTGAAATGAG-3'	
Beta Actin	Betaactin FOR	5'-CGTGCGTGACATTAAGGAGA-3'	**648bp**
	Betaactin Rev	5'-CACCTTCACCGTTCCAGTTT-3'	

Table 3: Reaction mixture for Real time PCR.

Components	Quantity (µl)
2X PCR Master Mix Syber Green	5
ROX	0.2
Forward primer	0.25
Reverse primer	0.25
cDNA	3
Distilled water	1.3
Total Kit	**10**

Statistical Analysis

Statistical analyses were performed with Graph Pad Prism 6.01 software. Results were expressed as the mean standard deviation (SD). Statistical differences were assessed by 2way Anova - Bonferroni's multiple comparison test and a value of p less than 0.05 was considered significant (Table 4A & 4B) (Figure 2).

Table 4A: Mean ΔCt of HSP 90 expression amongst the study groups.

Results	Normal	Inflamed	OLP
Mean	2.591	2.753	3.184
Standard Deviation	0.6943	0.8364	0.9117

Table 4B: Bonferroni's Multiple T-test Results p Value <0.05 was considered significant.

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significance	Summary
Normal vs. Inflamed	-0.1619	-0.3685 to 0.04467	Ns	0.87
Normal vs. OLP	-0.5934	-0.8000 to -0.3868	*	0.039
Inflamed vs. OLP	-0.4315	-0.6381 to -0.2249	*	<0.0005

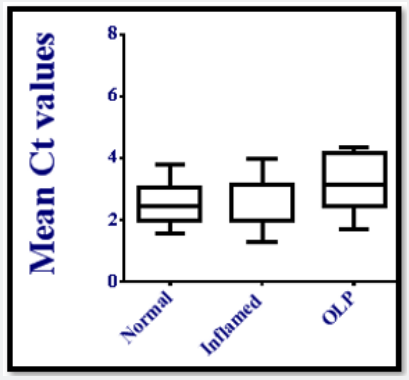

Figure 2: Depicts the Mean cT values of the expression of the protein amongst the study groups.

Effects on the Gene Expression

The levels of HSP 90 gene expression were measured by Real Time PCR. Changes in the expression levels of the protein between the study groups were normalized to beta actin levels and then calculated by Livak method. As the disease state progressed, the levels of HSP 90 gene expression was increasing accordingly. Real time PCR data analysis signified a gradual increase in the expression from Normal to inflamed and a sharp increase to OLP. The calculation method by which the fold changes are done have been elucidated in the Table 5. The fold changes in comparison to the normal is as shown in the Livak Method below. Using the formula, the fold change seen clearly proves the upregulation of the protein in OLP patients.

Table 5: Summary of fold change amongst the study groups in comparison to Normal.

Inflamed	OLP
1.12	1.51

Livak Method Formula

a. Normalize C_t (Target Gene) to C_t (Reference Gene) = ΔC_t (C_t Target -C_t Reference)

b. Normalize Δ C_t of case to C_t of Normal = $\Delta\Delta C_t$ (ΔC_tcase -ΔC_t normal)

c. Calculate expression ratio/fold change increase $2^{\Delta\Delta Ct}$

Application of the formula for calculating the fold change

Normal vs. Inflamed

a. Fold change in Normal vs. Inflamed

b. Mean ΔCt of Inflamed: 2.75

c. Mean ΔCt of Normal: 2.59

d. ΔΔCt: 2.75-2.59=0.16

e. $2^{\Delta\Delta Ct}$ Fold Change=$2^{0.16}$=1.117 (approx. to 1.12)

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Therefore, it is quantitatively estimated that the HSP 90 gene has a mean fold increase of 1.12 in the tissue samples of inflamed when compared to the normal samples using Real Time-PCR

Normal vs. OLP

a. Mean ΔCt of OLP: 3.18

b. Mean ΔCt of Normal: 2.59

c. ΔΔCt: 3.18-2.59=0.59

d. $2^{\Delta\Delta Ct}$ Fold Change = $2^{0.59}$ = 1.505 (approx. to 1.51)

Therefore, it is quantitatively estimated that the HSP 90 gene has a mean fold increase of 1.51 in the tissue samples of OLP when compared to the normal samples using Real Time-PCR

Discussion

Lichen planus is a chronic mucocutaneous disease of multifactorial etiology and pathogenesis. OLP is considered a potentially malignant lesion, so lesion monitoring must be periodic even in asymptomatic patients, and symptomatic ones should be treated. This chronic lesion is usually detected in 0.5-2.2% of population. Among these, only 0.5-.1.5% of the lesions progress to carcinoma. However, there are no prognostic markers available presently to recognize the increased risk in malignant transformation of the lesions. As well the current therapeutic regimen has the concept linking OLP and oral squamous cell carcinoma states that chronic inflammation results in crucial DNA damage, which further progresses to development of carcinoma. Even though in the past decade, enormous information has been accumulated on malignant potential of OLP, its transformation remains unclear. This molecular work done in identifying the role of HSP 90 in OLP as a good prognostic marker.

Corticosteroids are considered as first-line treatments since their topical form has better benefits and fewer side effects over time. Alternative therapies efficacy has not been demonstrated yet. These drawbacks make us identify the role of novel molecules that could be a potential target for the treatment of OLP. This study demonstrates and identifies HSP 90 as a novel bio-molecule. Heat shock proteins expressed by oral keratinocytes may be auto antigenic in Oral lichen planus. Susceptibility to Oral lichen planus may result from dysregulated heat shock protein gene expression by stressed oral keratinocytes or from an inability to suppress an immune response following self-Heat shock protein recognition. Over expression of Heat shock protein 90 in Oral lichen Planus has been associated to the persistence or chronicity of the disease, or they could have simply reflected cellular injury. The findings were substantiated with the study done by Bramanti TE, Yin Cao Shen Li Jia and Ponlatham, Yin Cao Shen, Li Jia.

Conclusion

The importance in identifying the role of HSP 90 in genomic analysis could provide rich information to help understand the pathology of a disease in an integrated way. Understanding the histology, premalignant state and molecular mechanisms of oral carcinogenesis ma Marzieh [13,14] facilitate the development of novel strategies for the prevention and treatment of oral cancers arising from Oral Lichen planus. This tumor-associated protein can be further evaluated as potential biomarker for pathogenetic investigations. To our best knowledge in the literature, there has not been any previous studies done in this sample size and tissue samples of OSCC and OLP Patients. This is the first global data to be published on this huge sample size. Though trials are currently happening in finding novel HSP 90 inhibitors, base for OSCC targeted therapy is still unknown and this study enlightens and unfolds the path to attempt HSP 90 in targeted therapy for OSCC patients. Studies on tissue samples are more authenticated and reliable as it directly is related to the patient and the results are validated better.

Ethics Statement/Confirmation of Patients' Permission

This study was approved by the Institutional Review Board, and all patients provided their informed consent for all examinations and experiments.

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