Regulation of Gene Expression by Quantitative Real Time PCR in Low Dose Isotretinoin Treated Acne Patients

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Abstract

Acne vulgaris is a common human skin disorder, hunting the younger age groups of people ranging from 10-35 years. It is not a deadly disease, but it is a major fact, that most of the suicides in adolescents are due to acne vulgaris. Accumulation of Propionibacterium acnes, sebum production, follicular hyper keratinisation and inflammation are some of the significant causes of pathogenesis of acne. Many medications including antibiotics like tetracycline, minocycline, erythromycin, clindamycin are given to the patients, but due to improper medication habit of the patient and certain resistance mechanisms of smart pathogens leads to the resistance of such bacteria against these antibiotics. Till date, isotretinoin and retinoic acid, are the best treatment for Acne vulgaris. This study was undertaken to analyze the regulation of genes expression after 1 and 8 weeks of isotretinoin (dose: 0.5 mg/kg/day) treatment given toacne patients. Upregulation in the expression of some prime genes like LCN2, KRT23, SERPINA3 accounts for the initiation of the immune response against the pathogens causing acne. Down regulation of genes like PDE6A, COL1A1, ALOX15B, MMP-2, INSIG1 etc. again demonstrates that the gene products which can convert sebum, fats and cholesterol to triglycerides would no further be beneficial for *P. acnes* inhabitation. The aim of this study was to understand the action of isotretinoinin the regulation of gene expression in acne patient.

Keywords: Acne vulgaris; Isotretinoin; *Propionibacterium acnes*

Introduction

Acne vulgaris is a common skin disorder having pernicious effects on millions of people worldwide. This disease not only affects the integrity of the skin, but also it has a very different effect on the mental health and esteem of the person suffering from acne. Many people commit suicide due to the lifelong scars mediated by this disease. It is more frequent [70-80%] and severe during the adolescence age [1-4]. Factors like increased sebum production, inflammation, follicular hyperkeratinization and the action of *P. acnes* within the follicle has been linked with the pathogenesis of acne vulgaris. Along with these, stress, food habits and genetic factors have been seen to be resposible in the development of acne.

Blocking of tiny holes in the skin called as hair follicles causes acne. The sebaceous glands are found near the surface of the skin and hair follicle grows out from there. These glands produces sebum which lubricates hair and skin and keeps them off from drying. When sebaceous glands starts producing more sebum, it mixes with dead skin cells and forms a plug in the follicle. This follicle if remains close to the surface of the skin, it bulges out forming whiteheads and if it becomes open to the skin, it forms blackheads. These plugged follicles gets contaminated by various skin commensal bacteria like *P. acnes*, *Staphylococcus aureus* etc. andincrease the severity of the disease [4].

Several therapies have been installed against acne till now but, none of them promises to be effective for a long time. Best example is the increasing resistance of acne causing bacteria against most of the available antibiotics like erythromycin, clindamycin and tetracycline, which are the first line of treatment against acne *vulgaris*. Therefore, there is an emerging need for a drug which can dodge bacterial efflux or resistance mechanisms for a longer period of time.

Since US Food and Drug Administration has approved oral isotretinoin in 1982, it has been seen to be the most efficient against acne compared to other treatment modals [1-5]. It influences on all of the major a etiological factors responsible for acne. The efficiency of this drug lies in its impact on cell-cycle progression, cellular differentiation, cell survival and apoptosis [6-11]. According to some news reports, responses of isotretinoin against acne, starts with 0.5 to 1.0 mg/kg for 4 to 6 weeks, but the treatment can take 16-20 weeks [12]. Use of isotretinoin not only affects the structural integrity of the skin, but it also alters the skin micflora [13]. Alteration in microflora is mainly acknowledged in terms of *P. acnes* population as it is considered as the causative organism of acne vulgaris. It is an anaerobic gram-positive bacteria which mainly resides in the pilosebaceous unit of the skin. As this unit is rich in sebum, triglycerides and lipids, it acts as a nutrient repository for the bacteria. This interaction leads to immunological reactions which further initiates release of cytokines (TLR2, IL-12, and IL-8), defensins and metalloproteinases causing aggravation in the diseased state. It has also been suggested that like all-trans-retinoic acid, isotretinoin might increase host defense mechanisms and modifies monocyte chemotaxis, which in part explains the anti-inflammatory effects of the drug [14-20]. Previous research work done on isotretinoin could not illustrate the mechanism of isotretinoin on acne but it has been seen that it reduces the size of sebaceous gland after 16 weeks of treatment. To decipher the exact pathway where isotretinoin acts to reduce acne progression, one must know those genes which get regulated after the treatment. Thus, if we could identify the initial changes going on, then it would be easier to narrow down those specific genes or pathways

which can be traced to target acne, hence on the basis of earlier studies, genes getting regulated within 1 and 8 weeks were selected for the study [21].

Materials and Methods

Patient selection

Skin Biopsy (3 mm punch) was collected from the back of patients suffering from severe acne, before and after 1 week and 8 weeks of isotretinoin treatment in Transport Media (Hank's Balanced Salt Solution with PenStrep) from Lok Nayak Jai Prakash Narayan Hospital, New Delhi. These patients were not under any kind of medication for the last two months. Demographic details of the patients are given in Table 1.

S.No.	Age	Sex	Dose of isotretinoin (mg/kg/d)	Biopsy taken at week
1.	20	M	0.5	1
2.	20	F	0.5	1
3.	20	M	0.5	1
4.	18	M	0.5	8

Table 1: Demographics of the patients.

Materials

Sterile eppendorfs containing chilled 500 µl of Transort Media (Hank's Balanced Salt Solution + 10% Anti-Anti) were used for keeping the biopsy taken from the back of acne patients. Dispase (Hi Media) enzyme of 2.4 U/ml was used to treat biopsy overnight at 40 C so as to remove epidermis from the dermis of the skin sample easily. RNA from the epidermis was isolated by Trizol (GIBCO) method. RNA was quantified in Nanodrop spectrophotometer. cDNA synthesis was done using Applied Biosystems cDNA kit, and Real time PCR was conducted on a 96 well Real time plate on Applied Biosystems 7500 Real time Fast PCR using Fast SYBR Mix procured from applied Biosystems. Primers for Real time PCR was made by using NCBI and Primer 3 tools, of the following genes: COL1A1, ALOX15B, LCN2, KRT23, DEFB1, IVL, SERPINA3, TMPRSS4, INSIG1, CHI3L1, MMP-2, PDE6A.

Methods

Skin biopsy (3 mm) from the back of the acne patient (18-20 yrs) was collectedin chilled transport media (HBSS) + 10% Anti-Anti) having PenStrep antibiotic combination to avoid bacterial contamination in the sample. Severe acne patients who hadnot taken any medication for the last two months were selected forbiopsy. Thebiopsy samples were collected before and after treatment with isotretinoin for 1 week and 8 weeks in the presene of a surgeon dermatologist. Biopsy samples were kept overnight at 40°C in mild dispase (2.4 U/ml) [22] enzyme to remove epidermis. Total RNA was isolated from the epidermis using TRIZOL (GIBCO) [23] and was quantified by a Nanodrop spectrophotometer. The RNA sample wasloaded on 2% agarose gel to analyze the quality of the isolated RNA. PCR with the negative control was set up with the isolated RNA against the housekeeping genes to ensure that RNA is not having any genomic DNA contamination. cDNA was made out of this RNA using reverse transcriptase enzyme and cDNA formed was further diluted in 1:5 ratio. Real time PCR of 10 µl reaction was performedon ABI 7500 real time fast PCR machine in a 96 well plate using Fast SYBR mix. Ct values were noted down for further calculation of fold change in the gene expression of the untreated andtreated isotretinoin in the acne samples using ddCT approach.

Results

In thisstudy, regulation in the expression of some major genes have been studied to understand the possible mechanism of action of isotretinoin. It wasinteresting to find that in all the three subjects, an upregulation of LCN2 and KRT23 genes is seen, as these are one of the imperative genes whose expression can control acne vulgaris Figure 1a. It was observed that after one week and 8 weeks of isotretinoin exposure, two genes (ALOX15B and COL1A1) and three imperative genes (PDE6A, ALOX15B and COL1A1 were downregulated respectively. Along with these, INSIG1, CHI3L1 and MMP-2 also are getting downregulated and is depicted in Figure 1b and 1d. SERPINA3 gene, which is a serine protease inhibitor, got upregulated on 1 week exposure. Figure 1c illustrated that in one of the subjects, IVL, DEFB1 and TMPRSS4 had upregulated, implying their role against *P. acnes* proliferation, inflammation and skin remodeling. Upregulation of these genes showed that even within a low dosage of this drug expression of genes can be regulated which may have activity against acne vulgaris.

Discussion

Oral isotretinoin has no direct antimicrobial action, but by dramatically reducing the size of the pilosebaceous duct it alters the microenvironment within the duct making it much less favorable for colonization with *P. acnes* [21,24,25]. A dose of 0.5–1.0 mg/kg/day radically reduces sebum excretion by the order of 90% within 6 weeks. But the size of sebaceous glands resumes its size within 2 months of non- exposure to isotretinoin. Some of the genes under expression study have been thought to lead us to the pathway through which this drug induces either very long remission or inhibition of acne vulgaris.

COL1A1 (Collagen, type1, alpha 1), gene encodes Type I which is a fibril-forming collagen found in most connective tissues and is abundant in bone, cornea, dermis and tendon. The downregulation of this gene after 8 weeks of treatment clearly illustrates that isotretinoin inhibits the collagen to accumulate in an around acne lesions, further inhibiting the comedones to form and protecting the skin integrity. PDE6A (phosphodiesterase 6A, cGMP-specific, rod, alpha), gene encodes the cyclic-GMP (cGMP)-specific phosphodiesterase 6A alpha subunit, expressed in cells of the retinal rod. The protein is a subunit of a key phototransduction enzyme and participates in processes of transmission and amplification of the visual signal. Mutations in this gene may lead to night blindness. This is one of the potential side effects of isotretinoin, and the downregulation of this gene here after isotretinoin treatment can serve as a major side effect along with treating acne vulgaris as it can induce changes in retinal expression [26].

The outermost layer of skin epidermis stratum corneum, is mainly composed of lipid and sterols [27] thus, downregulation of those genes whose product can metabolize lipids and sterols is an asset of using isotretinoin, because then *P. acnes* would be less exposed to its energy derieving factor and hence reducing comedogenesis. One such example is ALOX15B (Arachidonate 15-lipoxygenase, second type),

gene encodes a member of the lipoxygenase family of structurally related nonheme iron dioxygenases involved in the production of fatty acid hydroperoxides. The encoded protein converts arachidonic acid exclusively to 15S-hydroperoxyeicosatetraenoic acid. Its

downregulation on treatment with isotretinoin demonstrates that isotretinoin limits the nutrient supply to the bacteria residing in the acne lesions, especially to P.acnes, thus starving them to death.
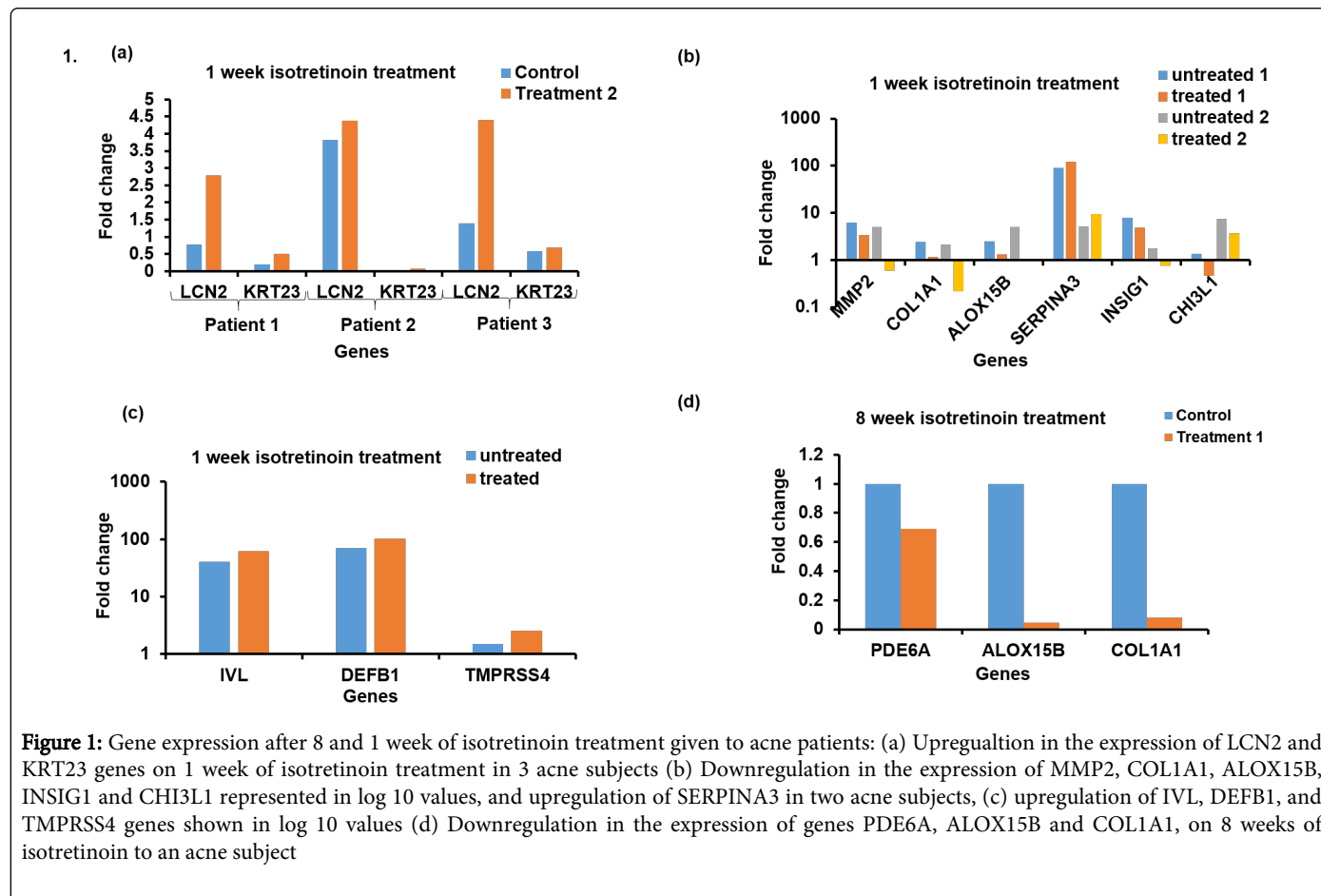
Figure 1: Gene expression after 8 and 1 week of isotretinoin treatment given to acne patients: (a) Upregualtion in the expression of LCN2 and KRT23 genes on 1 week of isotretinoin treatment in 3 acne subjects (b) Downregulation in the expression of MMP2, COL1A1, ALOX15B, INSIG1 and CHI3L1 represented in log 10 values, and upregulation of SERPINA3 in two acne subjects, (c) upregulation of IVL, DEFB1, and TMPRSS4 genes shown in log 10 values (d) Downregulation in the expression of genes PDE6A, ALOX15B and COL1A1, on 8 weeks of isotretinoin to an acne subject

A major gene involved in the interaction of isotretinoin with human genes is, LCN2. Expression of LCN2 is seen to be upregulated after exposure with isotretinoin for 1 week. Studies have shown that sterile wounding of healthy skin induces expression of antimicrobial peptides like defensins, LCN2 and protease inhibitors through activation of EGFR by heparin binding [28-30]. Thus, upregulation of LCN2 in treated patients exhibits that the LCN2 gene product has a role in captivating bacterial growth in acne patients. Earlier studies demonstrates increased susceptibility of bacteria due to the iron depleting strategy. Toll like receptors on immune cells stimulate the transcription, translation, and secretion of lipocalin 2 whenever they get exposed to bacterial accumulation. LCN2 then binds to the bacterial siderophores and kills the bacteria by making it iron deficient [29-31].

Also, there are some proteins which are very necessary to maintain the skin integrity like KRT23. Keratins are intermediate filaments forming units in epithelial cells. KRT23 belongs to acidic type 1 keratins. Gene array data showed upregulation of mRNA for keratins 7 and 19 when NHEK was treated with the different retinoids [32,33]. From the Figure 1(b), it is clearly noticeable that on 1 week treatment of isotretinoin to acne patients, the expression of KRT23 has upregulated indicating the role of isotretinoin in maintaining the skin integrity.

Human b-defensin 1 and 2 (DEFB1 and DEFB2) are expressed in the pilosebaceous unit and their expression is upregulated in acne lesions as a result of cutaneous innate immunity against *P. acnes* [34-41]. Here upregulation in the expression of DEFB1 in the patient implies that isotretinoin leads to increased inflammatory response, which can help in curbing growth of *P. acnes* and hence reducing the pathogenesis of acne vulgaris. Here in our study, quantitative PCR reflects the downregulation in the expression of MMP-2 after exposing the patients to isotretinoin for 1 week [42].

A study conducted by Nelson et al. shows that a significant proportion of these genes are involved in pathways that regulate differentiation, tumor suppression, serine proteases and serine protease inhibitors. TMPRSS4 is a serine protease of the family, chymotrypsin 1 and it has been seen to be overexpressed in pancreatic cancer [43,44]. It has a broad range of potential ligands like gram-positive and gram-negative bacteria and hence it can be annotated to have "pattern recognition receptor activity. These receptors acts as membrane-bound and soluble proteins which binds to pathogens and internalise microbes and their products including gram-positive bacteria (lipoteichoic acid), gram-negative bacteria (lipopolysaccharide), intracellular bacteria and CpG DNA. Its overexpression along with SERPINA3 after isotretinoin treatment for 1 week suggests their potential role in targeting the bacteria

responsible for acne vulgaris, suggesting their role in inducing an immunological response against pathogens [45,46].

There are certain gene products whose role are to maintain the lipid, fatty acids and matrix profile of the human skin. One of these genes under scrutiny was INSIG1. It restricts lipogenesis in mature adipocytes and blocks differentiation in preadipocytes. Thus, its downregulation in exp

ression, suggests that it can inhibit the nutrient supply to *P. acnes* by restricting lipogenesis [47]. Earlier studies have reported that gene expression of involucrin (IVL) increases during epidermal differentiation. It is synthesized in stratum spinosum and transglutaminase enzyme cross links it in the stratum granulosum rendering it highly stable. This strong envelope protects the skin from invasion of microbes [48-51]. According to the above said studies, if the expression of IVL is increasing in this study on isotretinoin treatment, we can infer that it would help our skin fight successfully against major acne causing pathogens. Protein of CHI3L1 is thought to have a role in tissue remodeling and inflammation. According to the study conducted by Nelson et al, 2009, isotretinoin decreases the expression of CHI3L1. Here in only 1 week of isotretinoin treatment to the patients it delivered a reduced expression of this product. Upregulation of this gene has been seen in many diseases associated with inflammation and fibrosis [51-54]. Therefore, its downregulation clearly demonstrates that it would further inhibit fibrosis around acne lesions and also reduced inflammation would decrease the severity of the disease.

Isotretinoin is till date the last resort for the acne patients, but along with treating acne, it also brings with itself some major adverse effects which need to be taken care of before and during medication. These side effects include gastrointestinal tract dyspepsia, vaginal candidiasis in women, and a small risk of photosensitivity. Dry lips, dry skin, dry eyes, decreased night vision, headache, epistaxis, and backache are some other effects to be added to the list. Certain elevation in liver enzymes and in serum lipid indices, especially triglycerides can also be seen in the acne patients under isotretinoin treatment. Along with these side effects, depression is also one of the issue, one should cater to. Thus, the dose and duration of treatment should be minimized to reduce the side effects.

This study shows the regulation of genes within one week of a minimum dose of isotretinoin treatment (0.5 mg/kg/day). Study of the expression of genes during a small time exposure to isotretinoin would help us understand better the pathways where it can act and those specific genes and pathways can be further targeted to control acne progression.

Acknowledgement

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