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Semi-Quantitative Gene Expression Studies of *Mmiβ* and *Skip* Involved in Endometrial Adenocarcinoma

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ABSTRACT

Skip gene encodes a protein which plays a very vital role in a number of metabolic pathways. It is very significant in the glucose metabolism where it regulates the signalling of the insulin pathway. But the full molecular details of the pathways regulated by *Skip* encoded protein are not known. It is speculated that *Skip* gene expression is altered in case of endometrial adenocarcinoma (EAC). *Mmiβ* gene encodes for a protein called Myosin-1c which acts an actin-based molecular motor in the cells. It has been studied that this gene down-regulates during endometrial adenocarcinoma and colorectal cancers. In this study the expression analysis of these two was carried out using multiplex PCR. An endogenous control was used for this PCR. *ACTB* gene served as the endogenous control because of it being a house keeping gene. It thus shows a universal expression in all cells. Thus in this study the gene expression of *Skip* and *Mmiβ* genes was comparatively analyzed with *ACTB* gene. The results that came out of this study showed an over-expression of *Skip* gene and down-regulation of *Mmiβ* gene with respect to *ACTB* gene in cancer cell lines as was indicated by the previous studies with these genes. Expression of both genes i.e., *Skip* and *Mmiβ* was statistically compared between normal and cancerous cell lines and was found statistically significant at a value of P.

Keywords: *Mmiβ*, *Skip*, gene expression, adenocarcinoma.

I. INTRODUCTION

Skip gene which encodes SKIP protein is located on 3rd sub-band of 13th band of short arm of chromosome 17 (17p13.3) in humans. This protein functions in numerous pathways such as, Inositol phosphate metabolism, Insulin signalling pathway. It is involved in processes such as, in response to insulin stimulus, glucose homeostasis, actin cytoskeleton organization, and in uteroembryonic development. The gene is 22.32 kb in length. SKIP (also known as D3zb21 or INPP5K), is a type II inositol polyphosphate 5-phosphatase that hydrolyses the inositol phosphates D5 position and also the corresponding phospholipids. An expression of 2 to 3kb transcripts of this gene is observed in all tissue samples when subjected to Northern blot analysis, with maximum expression in kidney, heart, and skeletal muscle. It was identified in insulin stimulated Chinese hamster ovary (CHO) cells that SKIP's ectopic expression inhibits phosphoinositide 3-kinase signalling. SKIP repressed downstream insulin target phosphorylation such as AKT, partly inhibited insulin-induced calcium mobilization. No modification was found in insulin-induced phosphorylation of mitogen-activated protein kinases (MAPKs) with SKIP association. Using antisense oligonucleotides for down-regulation of endogenous SKIP levels in its response to insulin elevated AKT phosphorylation. It is also studied that GLUT4 translocation inhibited by SKIP **[1]**. It has been seen that SKIP is associated with Epidermal Growth Factor (EGF) and Endoplasmic Reticulum (ER). When stimulated with EGF, SKIP is briefly translocated to plasma membrane and associates itself with sub-membranous actin **[2]**. *Mmiβ* gene which encodes Myosin-1c protein is located on 3rd sub-band of 13th band of short arm of chromosome 17 (17p13.3) in humans. This gene is 28.5 kb in length. Basically, a member of the unconventional myosin protein family is encoded by this gene, which is an actin-based molecular motor. This protein is found in the cytoplasm, but in the

nucleus a unique N-terminus isoform is also found which is associated with RNA polymerase I and II. It also has a role in initiation of transcription. The protein contains 1063 amino acids, which contains actin-binding and ATP-binding sequences in its motor domain. MMIb protein (also known as myosin I-beta and MYR2), is believed to be involved in the mediation towards the slow part of adaptation by hair cells which are the sensory cells of the inner ear **[3]**. In phosphatidylinositol-3-hydroxykinase (PI3K)-independent insulin signalling pathway, the role of MMIb is to control the movement of vesicles containing intracellular GLUT4 towards the plasma membrane **[4]**. *ACTB* gene, a housekeeping gene is located on 22nd band of short arm of chromosome 7 (7p22) in humans. The gene is 3.45 kb in length. Beta-actin (*ACTB* gene) is among the six diverse isoforms of actin identified in humans and it is one of the two non-muscle cytoskeletal actins, which are highly conserved proteins. Their involvement in cell motility, structure and integrity are well known. It is known that in wild type as well as mutant cells beta-actin was stable and does not undergo degradation by any means. Arginylation of beta-actin was seen to regulate cell motility **[5]**. If beta-actin mRNA was localized to the sites of active actin polymerization, it leads to the control of differentiation, embryonic cell migration, and probably carcinogenesis. For this type of localization it needs the help of an onco-fetal protein ZBP1 which binds to the 3-prime untranslated region of the beta-actin mRNA which is a conserved 54-nucleotide element known as the 'zipcode.' In primary fibroblasts and neurons, ZBP1 stimulates translocation of the beta-actin transcript to actin-rich protrusions most probably helping in cell migration and neurite outgrowth. When beta-actin transcript associates with ZBP1 in the nucleus, it blocks translation initiation thus inhibiting and avoiding premature translation in the cytoplasm. Cell proliferation, vesicle trafficking, and secretion is regulated by the interaction of phospholipase D with actin microfilaments **[6]**. To check the gene expression levels between normal and pathological samples, the most significant difficulty is the variation in the amount of genetic material between these samples which is mainly due to fluctuations in the efficiency of reverse transcription, which ultimately affects mRNA isolation. So it becomes necessary to normalize the output from the samples. So to check the expression of a gene or genes in normal as well as cancerous cell lines a reference gene is used. This criterion is fulfilled by Housekeeping genes (HKGs) and that is why they are used for normalization purposes in most of the expression studies **[7]**. The most common housekeeping genes which are used in almost all semi-quantitative multiplex PCR studies as a loading control are *ACTB* (Beta Actin) and *G3PD* (glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*]) **[8]**. In order to swiftly identify deletions or duplications in a large gene a Multiplex polymerase chain reaction (Multiplex PCR), a variation of PCR has been developed. In this process the genomic DNA is amplified in a thermal cycler by using multiple primers and temperature mediated DNA polymerase (Taq Polymerase). There are multiple primers in a single PCR mixture which leads to the production of varying sizes of amplicons which are particular to different DNA sequences. Nevertheless for achieving near to perfection, optimization of annealing temperatures of the primers should be done and also amplicon sizes should be of varying base pair length so that they form different bands during gel electrophoresis. The aim of the research in progress was to analyze and infer the gene expression of *Mmiβ* and *Skip* in endometrial carcinoma cancer cell lines. Ten different type of cell lines were used to study expression of both genes, five of which were normal cell lines and the other five were cancerous cell lines.

II. METHODS AND MATERIAL

In vitro cell culture conditions

Primary cell cultures were established from endometrial adenoma cancer tumors (EAC) which were grown on DMEM media supplemented with non-essential amino acids [1:100] (Gibco, USA), 1 mM sodium pyruvate (Gibco, USA), 50 µg/ml Pen-Strep (Gibco, USA) and 10% fetal bovine serum [FBS] (Gibco, USA), for 5-7 passages in order to obtain maximum number of cells. NME cells lines were cultured under same conditions, but unlike EAC medium for NME contained 15% FBS. The cells were grown at 37°C in an atmosphere of 90% humidity and 5% CO2 and detached with 0.25% Trypsin-EDTA (Gibco, USA) at 37°C for 15 min at a confluence of 70-80%.

RNA extraction

Total RNA was extracted from cells grown on 6-well plate at $3x10^5$ cells overnight at 37°C in complete DMEM medium supplemented with 5% FBS using RNeasy Mini kit (QIAGEN) and treated with DNAse I

(QIAGEN). cDNA was synthesized from mRNA of these 10 different cell lines using the Superscript III first strand Reverse Transcriptase PCR system (High Capacity RNA to cDNA kit, Applied Biosystem) with oligo-dT primers (Thermoscientific).

Multiplex PCR amplification

Skip, *Mmiβ* and *ACTB* genes from various cDNA samples obtained from ten different cell lines of which five were Endometrial Adenocarcinoma (EAC) while five were Non Malignant Endometrium (NME) samples, which were amplified by means of PCR. The pre-PCR mix contained *Skip*, *Mmiβ* and *ACTB* genes and dNTP mix (Thermoscientific). Moreover the mix also contained Red Hot DNA Taq polymerase (Thermoscientific), PCR reaction buffer and $MgCl_2$. By using autoclaved water the volume of the master mix was made up 24µl. 11 different PCR tubes marked from 1 to 11 were made. All 11 tubes except the last one was arranged in such a way that one corresponded to cancerous cell line while the other was that of a normal cell line. After this 1 µl of cDNA sample obtained from ten different cell lines was added in each PCR tube respectively. In the 11th tube, instead of cDNA equal amounts of autoclaved water was added. This 11th tube functioned as a negative control. The samples were then subjected to PCR (TaKaRa PCR Thermal Cycler Dice) for 27 cycles.

Initial Denaturation	94°C	3 minutes	
Denaturation	94°C	1 minute	25-28 cycles (27 cycles used)
Annealing	55°C	1 minute	
Extention	72°C	30 seconds	
Final Extention	72°C	7 minutes	

Agarose gel electrophoresis

Subsequently, after the PCR, the post-PCR samples were subjected to agarose gel electrophoresis. A 2% gel was made in which Ethidium Bromide (EtBr), which acts as an intercalating agent between the DNA strands making it easy to visualize the product on the gel. In the meantime, an electrophoretic gel apparatus with the combs was set. The gel was then poured into it and let it to solidify. In the period in-between, the loading sample mixes were prepared for gel electrophoresis. For this, afresh set of 11 PCR tubes were taken, in each of which 2µl of autoclaved water and 3µl of 5X Loading Dye was added. From each PCR sample 5µl of the PCR product was added into the new tubes and they were mixed by pipetting. The gel-run samples hence prepared were loaded into the wells of the gel. The first well was loaded with Ladder (Super Ladder Stage 100 bp, Thermoscientific). Samples were added such that cancerous as well as normal cell line samples were loaded in the wells in an alternative pattern. The samples were run inside the electrophoretic unit at 95 volts. After subjecting the samples to electrophoresis, the gel was visualized by ultraviolet gel imager (GE® ImageQuant ™ LAS 4000) and then the image results were examined by Image Quant 4000 (GE®).

Optimization and visualization

Numerous trials were performed to optimize the results. Optimizations were carried out in two phases – Primarily, optimization of the samples was carried out prior to PCR. The PCR experiments were carried out three times respectively. For the final optimization, both *Skip* and *Mmiβ* primer concentrations were also increased. At last, the cDNA samples for five different cell lines that is, Nut 6 (1st Tube), Nut 12 (3rd Tube), Nut 62 (4th Tube), Nut 14 (5th Tube) and Nut 100 (7th Tube) were increased in a range between 1.5µl to 2.5µl. Secondarily, optimization of the samples was carried out prior to electrophoresis. For optimization different gel runs were performed by making new loading sample mixes by changing the concentration of the PCR sample mixture which was increased or decreased depending upon the lighter or brighter band results previously obtained. Also in each sample the volume of the autoclaved water to be supplemented was altered, increased or decreased depending upon the brighter or lighter bands obtained previously. But in all samples the volume of the loading dye was kept constant.

Statistical analysis and inference by ImageJ

The visualization results obtained from Image Quant 4000 (GE®) were inverted using the same software by which the bands were changed to dark black while the background was changed to light grey. Later a software namely ImageJ 1.51j8 (Wayne Rasband, National Institute of Health, USA) was used for analyzing and inferring the results obtained from inverted gel image.

All values were statistically compared between normal and cancerous cells using student t-test.

III. RESULTS AND DISCUSSION

With respect to the endogenous control that is *ACTB* gene, expression levels of concerned gene, *Skip* and already functionally-known gene, *Mmiβ* were investigated by performing the multiplex PCR for ten different samples. Experiments in triplicates were run and optimized for the results. For achieving this, the primer concentrations (excluding the *ACTB* primer concentration) as well as cDNA concentrations of some of the cell lines were changed in order to get better results with bands of even strength. The genes *ACTB*, *Mmiβ* and *Skip* used were of the size 375 bp, 182 bp and 150 bp respectively. In the beginning trials only two bands were seen which when correlated with the ladder were *ACTB* – 375 bp, top band and *Mmiβ* – 182 bp, intermediate band, but no trial mentioned showed the band for *Skip* – 150 bp, lower band when the gel was visualized. The lack of *Skip* bands could possibly be because of two reasons. First, primer concentration of *SKIP* would have been less for setting off polymerization reaction. Second, the *Skip* gene primers used for initiating polymerization reaction would have been to some extent contaminated (that is either ionic or phenolic) or inappropriate sequence length which sometimes may perhaps lead to cross-priming reactions hence forming primer-dimers **[9]**. Nevertheless, no bands were seen in the samples containing cDNA of Nut12, Nut62, Nut14 and Nut100 cell lines. Somehow either the cDNA concentration of these cell lines was too low or the cell lines itself did not work. The best possible and very recurrent reason behind the absence of bands in certain cell lines could be because of some Taq polymerase inhibitor which could have contaminated the cDNA of these particular cell lines. Appropriate amplification during PCR is hampered chiefly due to diminutive contaminations which also have some Taq Polymerase inhibitor **[10]**. Afresh master mixes were made for PCR trial in order to optimize the entire samples by judging on the previous trial results. In this trial the *Skip* primer volume was increased so as to get proper three bands after gel electrophoresis and likewise the cDNA of the cell lines Nut6, Nut12, Nut62, Nut14 and Nut100 were increased so as to make more target DNA accessible for PCR amplification. All samples showed clear and bright bands for all three genes. Although very faint, *Skip* bands were seen. In Nut14 and Nut62 cell lines the bands were very much faint. So, another experiment was carried out in which the *Mmiβ* as well as *Skip* primers were increased so as to get brighter bands after gel electrophoresis and this time the cDNA of the cell lines Nut12, Nut62, Nut14 and Nut100 were further increased so as to get good bands in these cell lines also. Now, all samples showed clear and bright bands for all three genes, although very faint bands were seen in the cell lines with increased cDNA content. Three finest results obtained from Image Lab 3.0 (BioRad®) were selected and inverted using the same software. Later on ImageJ 1.44p (Wayne Rasband, National Institute of Health, USA) was executed for analyzing and inferring the results obtained from inverted gel image (Figure 1).

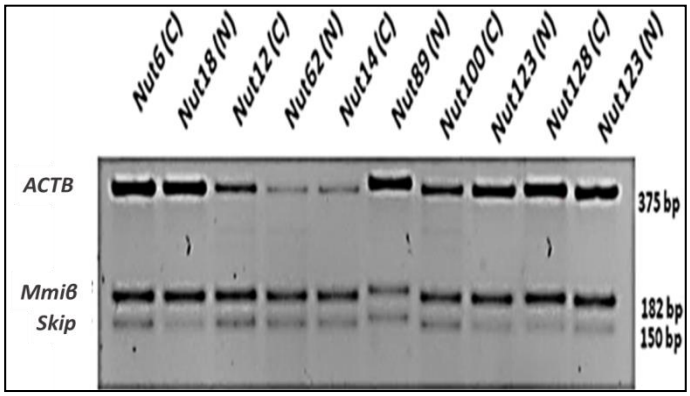

Figure 1 : Representative image from three replicates for gene expression of *Mmiβ* and *Skip* for 10 different EAC cell lines.

Mmiβ and *Skip* gene values were normalized with that of *ACTB* gene values respectively for EAC as well as NME samples. Normalization was done for three different independent results so that they could be inferred accurately. Then EAC was tallied with NME first for *Mmiβ* gene and then for *Skip* gene. Final mean was taken from all the three normalization values of *Mmiβ* and *Skip* gene respectively. A graph was plotted from these final mean values of these two genes. Standard deviation among mean of the three replicates were shown in the form of standard bars. The final results were then analyzed and inferred for whether the two genes have up-regulated or down-regulated with respect to *ACTB* in EAC (C) with respect to NME (N).

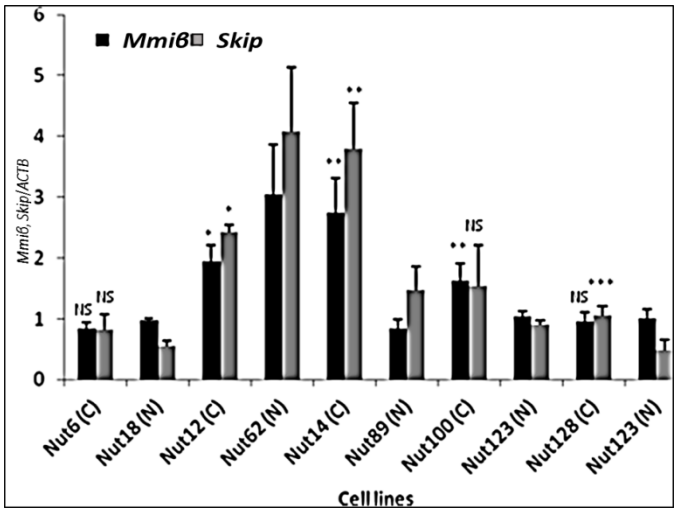

Figure 2: Expression of *Mmiβ* and *Skip* profile in five normal and five cancer cells. Expression of both genes were normalized with *ACTB*. Results are shown as mean from three different experiments while bar represents standard deviation among replicates. Level of significance among normal and cancer cells is shown in terms of p-value (* for p<0,1, ** for p<0.01 and *** for p<0.001).

Taking all the three independent values into consideration, the summed up results give a broader outcome about the expression of *Mmiβ* and *Skip* genes in the cancer cell lines as compared to the normal cell lines. In the summed up result and its graph mentioned above it is clearly seen that in almost all cancer cell lines *Mmiβ* gene is down-regulated while *Skip* gene is up-regulated. Unanimously, it can be inferred in case of caner that the *Mmiβ* gene shows an overall down-regulation, while *Skip* gene shows an overall up-regulation. In case of *Skip* gene abnormal expression levels is examined in case of cancer cells. Therefore, as inferred by the results, it is observed that *Skip* gene is up-regulated in cancer cells unlike *Mmiβ* gene, from which it is concluded that *Skip* may be a potential gene which is involved and may lead to Endometrial Adenocarcinoma (EAC).

IV.CONCLUSION

Analysis of the expression of the genes *Mmiβ* and *Skip* was carried out with multiplex PCR. *ACTB* which is a house keeping gene and hence shows constant expression was used as the endogenous control. As per the results got, *Skip* gene showed greater expression in case of endometrial adenocarcinomic cells as was supposed. It is also concluded from this study that *Mmiβ* gene down-regulates in the cancer cells as has been shown by the previous work. The action at the molecular level of *Skip* gene can be known thus elucidating the involvement of this gene in various signaling cascades which get active during endometrial adenocarcinoma. Apart from it, this study can prove useful to know further involvement of certain other genes in cancer.

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