International Conference on Medical Ethics and Professionalism

(ICMEP-19)

Dubai, UAE

23rd February, 2019

IARF Conference

www.iarfconference.com
Editorial:

We cordially invite you to attend the International Conference on Medical Ethics and Professionalism (ICMEP-19), which will be held in Dubai, UAE on February 23rd, 2019. The main objective of ICMEP-19 is to provide a platform for researchers, engineers, academicians as well as industrial professionals from all over the world to present their research results and development activities in Medical Ethics and Professionalism. This conference provides opportunities for the delegates to exchange new ideas and experience face to face, to establish business or research relations and to find global partners for future collaboration.

These proceedings collect the up-to-date, comprehensive and worldwide state-of-art knowledge on Medical Ethics and Professionalism. All accepted papers were subjected to strict peer-reviewing by 2-4 expert referees. The papers have been selected for these proceedings because of their quality and the relevance to the conference. We hope these proceedings will not only provide the readers a broad overview of the latest research results on Medical Ethics and Professionalism but also provide the readers a valuable summary and reference in these fields.

The conference is supported by many universities and research institutes. Many professors played an important role in the successful holding of the conference, so we would like to take this opportunity to express our sincere gratitude and highest respects to them. They have worked very hard in reviewing papers and making valuable suggestions for the authors to improve their work. We also would like to express our gratitude to the external reviewers, for providing extra help in the review process, and to the authors for contributing their research result to the conference.

Since December 2018, the Organizing Committees have received more than 40 manuscript papers, and the papers cover all the aspects in Medical Ethics and Professionalism. Finally, after review, about 11 papers were included to the proceedings of ICMEP-19.

We would like to extend our appreciation to all participants in the conference for their great contribution to the success of International Conference 2019. We would like to thank the keynote and individual speakers and all participating authors for their hard work and time. We also sincerely appreciate the work by the technical program committee and all reviewers, whose contributions make this conference possible. We would like to extend our thanks to all the referees for their constructive comments on all papers; especially, we would like to thank to organizing committee for their hard work.
Acknowledgement

IARF is hosting the International Conference on Medical Ethics and Professionalism this year in month of February. Technical advantage is the backbone of development and Medical Ethics and Professionalism has become the platform behind all the sustainable growth International Conference on Medical Ethics and Professionalism will provide a forum for students, professional engineers, academician, and scientist engaged in research and development to convene and present their latest scholarly work and application in the industry. The primary goal of the conference is to promote research and developmental activities in Public Health and to promote scientific information interchange between researchers, developers, engineers, students, and practitioners working in and around the world. The aim of the Conference is to provide a platform to the researchers and practitioners from both academia as well as industry to meet the share cutting-edge development in the field.

I express my hearty gratitude to all my Colleagues, Staffs, Professors, Reviewers and Members of organizing committee for their hearty and dedicated support to make this conference successful. I am also thankful to all our delegates for their pain staking effort to travel such a long distance to attain this conference.

Dr. Simpson Rodricks
President
IARF Conference (IARF)
<table>
<thead>
<tr>
<th>S.NO</th>
<th>TITLES AND AUTHORS</th>
<th>PAGE NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Skin Texture Analysis for the Chronic Kidney Diseases Patient</td>
<td>1 – 4</td>
</tr>
<tr>
<td></td>
<td>➢ U.Madhanlal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Dr.R.Kalpana</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Dr.P.Soundararajan</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Phenotype Frequencies of Blood Group Systems (ABO, Rh, Kell) Among Deferent Patients In Jaipur India</td>
<td>5 – 8</td>
</tr>
<tr>
<td></td>
<td>➢ Ziad Amran</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Manju Mhera</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Pankaj Agarwal</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Adeno-Associated Virus (AAV)-2 Genome in Arthrobacter sp. LS16?</td>
<td>9 – 10</td>
</tr>
<tr>
<td></td>
<td>➢ Arumugam S</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Jayandharan GR</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Folate Metabolism and Genetic Variant in Down Syndrome: A Meta- Analysis</td>
<td>11 – 16</td>
</tr>
<tr>
<td></td>
<td>➢ Ambreen Asim</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Sarita Agarwal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Sakil Subhash Kulkarni</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Inusha Panigrahi</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Hallermann Streiff Syndrome-The Oral Manifestations in a Child</td>
<td>17 – 20</td>
</tr>
<tr>
<td></td>
<td>➢ Sonu Acharya</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Manta Mohanty</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Sheetal Acharya</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>PARP1 Differentially Interacts with Promoter region of DUX4 Gene in FSHD Myoblasts</td>
<td>21 – 31</td>
</tr>
<tr>
<td></td>
<td>➢ Vishakha Sharma</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Sachchida Nand Pandey</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Hunain Khawaja</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Kristy J Brown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Yetrib Hathout</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Yi-Wen Chen</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Prenatal Assessment of Three Rare Syndromes from Telangana Region by 3D/4D Sonography</td>
<td>32 – 35</td>
</tr>
<tr>
<td></td>
<td>➢ MLN Deepika</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ T Sunitha</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ B Srinadh</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ K Rebekah Prasooona,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ M Sujatha</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Aruna Ramaiiah</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ A Jyothy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ Pratibha Nallari</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➢ KV Venkatachalam</td>
<td></td>
</tr>
<tr>
<td>S.NO</td>
<td>TITLES AND AUTHORS</td>
<td>PAGE NO</td>
</tr>
<tr>
<td>------</td>
<td>--------------------</td>
<td>---------</td>
</tr>
<tr>
<td>9.</td>
<td>Role of Genetic Testing in Lung Transplantation; Prediction of Inflammation</td>
<td>38 – 40</td>
</tr>
<tr>
<td></td>
<td>Mohamed SA Mohamed</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>Toll-like receptors and calcium-activated chloride/potassium channels as Immune modulators of allergic airway inflammation and asthma: a public health Research experience in State of Nebraska, USA</td>
<td>41 – 43</td>
</tr>
<tr>
<td></td>
<td>Saumya Pandey</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Antimicrobial activity of Terminalia bellerica (Gaertn.)Roxb. Against Multi Drug Resistant Staphylococcus aureus</td>
<td>44 – 50</td>
</tr>
<tr>
<td></td>
<td>Tamalika Chakraborty</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sumana Chatterjee</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lopamudra Datta</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abhijit Sengupta</td>
<td></td>
</tr>
</tbody>
</table>
Skin Texture Analysis for the Chronic Kidney Diseases Patient


[1][2] Department of Biomedical Engineering, Rajalakshmi Engineering College,

[3] Department Of Nephrology, Sri Ramachandra Medical College and Research Institute

madhanlal.34@gmail.com, Kalpana.r@rajalakshmi.edu.in, hod.nephrology@sramachandra.edu.in

Abstract—Assessment of kidney function is important, specifically for subjects with diabetic. Presently it is determined through Glomerular Filtration Rate (GFR) that involves parameters like age, serum creatinine, and weight, height, gender, albumin and skin color. In general, kidney dysfunction leads to change in fluid secretion in the body. Envisaging this changes could have an impact on properties of skin, attempted here is to find the changes in the skin through its texture properties. Hence skin images are obtained from Chronic Kidney Diseases (CKD) patients. These images are enhanced using watershed segmentation procedure and the lines formation are extracted using gradient based technique. Then the textural features like contrast, correlation, energy, homogeneity are evaluated for all the subjects. When the present GFR is modified using these textural features, the obtained new GFR values are agreeable with GFR values obtained from standard algorithms. Hence, it could be concluded that, kidney dysfunction has an influence on skin that could be quantified in terms of texture values.

Index Terms—Glomerular Filtration Rate (GFR), Chronic Kidney Diseases (CKD), Watershed segmentation, Gradient based technique, Textural feature.

I. INTRODUCTION

Stratifying and identifying patients at risk for renal disease are integral parts of clinical nephrology. This is accomplished by computation of Glomerular Filtration Rate (GFR), which is consider to be one of the reliable indicator of renal function. The GFR can be detected by measuring the filtration markers insulin, (25I) iothalamate,99mTcdiethylenetriaminepentaacetic acid, and iohexol [1, 2]. However, because these markers are, to varying degrees, costly and cumbersome to use and which leads to radioactivity, also necessitates special handling and disposal, therefore this kind of measurement is not used unless the situation warrants.

A far more common method to estimate renal function by finding the rate of blood filtration at glomerulus using GFR, that is computed based on demographic characteristics, such as age, gender, race, height, weight, and biochemical indices, including serum creatinine, urea, blood urea nitrogen (BUN) [3, 4]. Modification of Diet in Renal Disease (MDRD) equation and Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation are two method commonly used to predict of CKD [5].

II. DATA ACQUISITION

The standard demographic characteristics and the biochemical indices are obtained from 10 subjects in the age group between 12 and 80 years (9 diagnosed for CKD at different stages and remaining 1 is normal).

To quantify the skin tone, skin images are also obtained from the entire subjects using Galaxy Tab A with the resolution of 2592 x 1944. The images are acquired at normal room temperature with standard lightning arrangement. All the images are acquired from the lateral position of the fore hand.

III. LINE DETECTION

In order to extract the textural feature of the CKD patient skin filtration and image segmentation is done by morphological parameter using watershed segmentation.

After enhancing the image in order to view the textural changes and direction gradient is applied to the image.

The gradient is used to compute directional gradients, Gx and Gy, with respect to the x-axis and y-axis. The x-axis is defined along the columns going right and the y-axis is defined along the rows going down. The gradient magnitude and direction are then computed from their orthogonal components Gx and Gy.
The gradient of the image is given by the equation,
\[
\nabla = \begin{bmatrix} \frac{\partial f}{\partial x} \\ \frac{\partial f}{\partial y} \end{bmatrix}
\]
(1)

Where,
\[\frac{\partial f}{\partial x}\] is the gradient in the x direction.
\[\frac{\partial f}{\partial y}\] is the gradient in the y direction.
The gradient direction can be calculated by,
\[
\theta = \tan^{-1}\left(\frac{\partial f}{\partial y} \frac{\partial f}{\partial x}\right) \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots (2)
\]
Sample of line detection results are presented in Fig.1. and Fig.2.
The texture analysis is carried out to quantify the characteristic of the human skin texture by approaching image processing using Grey Level Co-Occurrence Matrix. GLCM is determined by the maximum grey value of the pixel and the number of grey-levels adopted. It is crucial to determine the efficacy of GLCM computation. More levels would mean more accurate depiction of textural information, but it also implies more computational burden [16].

### IV. PARAMETERS EXTRACTED

In this study, we only use and compute the following four main Hurlock’s coefficients on GLCM for texture analysis and the classification: Contrast (Cont), Energy, Correlation (Corr) and Homogeneity (Hgt) parameters. The correlation feature based on the variance (\(\sigma\)) and Mean parameter (\(\mu\)). The coefficient \(\sigma\) is specified by the following equations:
\[
\sigma = \sqrt{\sum_{i=1}^{Ng} \sum_{j=1}^{Ng} (1 - \mu)^2 \cdot P_{d,r}(i,j)} \ldots \ldots \ldots \ldots (3)
\]
\[
\mu = \sum_{i=1}^{Ng} \sum_{j=1}^{Ng} (i) \cdot P_{d,r}(i,j) \ldots \ldots \ldots \ldots \ldots \ldots \ldots (4)
\]
GLCM extracted the Haralick’s parameters from GLCM matrix of each cell segmented. These coefficients are listed below;
The Contrast (Cont) feature used to measure the image intensity contrast or the local variations present in an image to show the texture fineness. This parameter is specified by the following equation:
\[
\text{Cont} = \sum_{i=1}^{Ng} \sum_{j=1}^{Ng} (1 - j)^2 \cdot P_{d,r}(i,j) \ldots \ldots \ldots (5)
\]
The Energy feature returns the sum of squared element in the GLCM and they are expressed by the following equation:
\[
\text{Energy} = \sum_{i=1}^{Ng} \sum_{j=1}^{Ng} P_{d,r}(i,j)^2 \ldots \ldots \ldots (6)
\]
The Correlation (Corr) measures the linear dependence of gray level values in the co-occurrence matrix and describes the correlations between the rows and columns of the co-occurrence matrix. This parameter is specified by the following equation:
\[
\text{Corr} = \sum_{i=1}^{Ng} \sum_{j=1}^{Ng} (i - \mu_x)(j - \mu_y) \cdot \frac{P_{d,r}(i,j)}{\sigma_{xy}} \ldots \ldots \ldots (7)
\]
Homogeneity (Hgt) used to returns a value that measures the closeness of the distribution of elements in the GLCM to the GLCM diagonal. This coefficient is specified by the following equations:
\[
\text{Hgt} = \sum_{i=1}^{Ng} \sum_{j=1}^{Ng} \frac{P_{d,r}(i,j)}{(1 + |i - j|)} \ldots \ldots \ldots \ldots (8)
\]

**Fig 1. Skin texture analysis of CKD patient a) Original image b) enhanced image c) gradient directional image.**

**Fig 2. Skin texture analysis of normal people a)Original image b) enhanced image c) gradient directional image.**

### V. GFR COMPUTATION

GFR using standard formula like MDRD and CKD-EPI are computed for all the 10 subjects.
- MDRD formula: \(175 \times S_{cr}^{-1.154} \times \text{age}^{-0.203}\) for men
- MDRD formula: \(175 \times S_{cr}^{-1.154} \times \text{age}^{-0.203} \times 0.742\) (if female) \times 1.212 (if black)

The CKD-EPI Equations used for calculating the GFR rate is listed below,
- CKD-EPI = \(141 \times \min(S_{cr}/k,1)^{1.209} \times \max(S_{cr}/k,1)^{1.209} \times 0.993^{0.018}\) (female) \times 1.159 (black)

IARF International Conference Dubai 2 ISBN: 9788192958093
Skin Texture Analysis for the Chronic Kidney Diseases Patient

K=0.7 female  
K=0.9 male

α=-0.329 female  
α=-0.411 male

MDRD equation is modified as given below and is proposed to be a new method of computing GFR.

\[ \text{New GFR} = 175 \times S_{cr}^{1.154} \times \text{age}^{-0.203} \times \text{Hgt} \]

GFR are now computed for all the 10 subjects using the above mentioned 3 equations (2 standard equations and 1 new equation) is presented in the Fig 3.

![Fig 3. Variation of GFR against creatinine](image)

VI. CONCLUSION

The sole aim of the work is to find the relationship between renal function and to find skin tone. Hence, skin images are obtained from different subject, after preprocessing, textural feature are obtained by replacing 2 demographic parameter in the standard MDRD equation with the textural parameter, a new equation is formed. GFR value obtained through the proposed method tallies with the standard method. Hence it can be concluded that gender and race could be quantified in terms of textural value, which would facilitate still more accurate computation of GFR for different races of people. Skin manifestations occurring exclusively in the patient with altered renal function has already established [11-13]. The result of this study also establishes the fact of change in skin occurring in renal disorder subject.

REFERENCES


[8] Andrew D. Rule MD; Timothy S. Larson, MD; Erilik J. Bergstrahl, MSC; Jeff M. Slezk, MSC; Steven J. Jacobsen, MD, PHD; and Fernando G. Cosio, MD (December 2004) ‘Using Serum Creatinine To Estimate Glomerular Filtration Rate : Accuracy in Good health and in chronic kidney disease’-Annals of internal medicine 141,12 proquest medical library.

[9] Andrew S. Levey, MD; Lesley A. Stevens, MD, MS; Christopher H. Schmid, PhD; Yaping (Lucy) Zhang, MS; Alejandro F. Castro III, MPH; Harold I. Feldman, MD, MSCE; John W. Kusek, PhD; Paul Eggers, PhD; Frederick Van Lente, PhD; Tom Greene, PhD; Josef Coresh, MD, PhD, MHS, CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) (May 2009) ‘A New Equation to Estimate Glomerular Filtration Rate’ Ann Intern Med.; 150(9): 604–612.


Phenotype Frequencies of Blood Group Systems (ABO, Rh, Kell) Among Different Patients in Jaipur, India


[1] Educational level/Academic degree: (Ph.d Research Scholar) in Medical Lab Technology-Pathology, NIMS Medical College and Hospital, Jaipur, India
[2] Educational level/Academic degree: Prof. (Dr.) Pathology NIMS Medical College and Hospital /Pathology Department, Jaipur, India
[3] SDM Hospital, Jaipur, India. Pin Code-302002 Educational level/Academic degree: Prof (Dr.) Microbiology

Abstract—Background: With the industrial revolution of modern equipment and preparation of reagents, but in most blood banks; blood transfusions are done based on ABO & Rh antigens for pre-transfusion compatibility testing and achieved by random cross-matching of available units in the inventory storage. Unknown phenotype blood group for clinically significant antigens may cause in immunization. Aim: To determine the frequency of ABO, kell, Rh blood grouping, and antigens testing and antigen profile.

Materials and Methods: A total of 103 samples were collected from different patients at NIMS and SDM Hospital in Jaipur, India. Laboratory investigations were done on all the studied patients including: ABO, Rh blood grouping and Du testing have done by (BioVue cassettes based on Column Agglutination Technology), finally antigen profile (Rh; Kell Phenotype) by DiaMed ID-Card (C-e-E-e-K-Ctl, Monoclonal). Results: Total of 103 patients of different diseases 54 (52.5%) were males and 49 (47.5%) were females. The prevalence of Rh positive was 82.5%. The most common in distribution of ABO group system was B group (40.5%) followed by O (26.5%) then A group (23%). The most antigen frequent was antigen (e: 87.4%) and followed by D: 82.5%, C: 70.8%, c: 65%, E: 11.6%, while kell antigen was (2.9%). Conclusion: Our study, first step to make a rare donor antigens database bank and patients’ antibodies also, to provide compatible blood to all multiple blood transfused, alloimmunized patients and enhance safety in transfusion medicine.

Keywords: Phenotype frequency; ABO; Rh; Kell; Rajasthan.

I. INTRODUCTION

With the industrial revolution of modern equipment and preparation of reagents, but in most blood banks; blood transfusions are done based on ABO & Rh antigens for pre-transfusion compatibility testing and achieved by random cross-matching of available units in the inventory storage.1,2,3 But unknown phenotype blood group for clinically significant antigens may cause in immunization especially in multiple blood transfused patients as thalassemia, sickle cell disease and chronic renal failure.4 There up to 305 red blood cell antigens are known, and clustered into 35 blood grouping, nine of which are considered to be main blood group (ABO, Rh, Kell, Kidd, Duffy, MNS, P, Lewis, and Lutheran). 5,6 Information about all blood groups antigens frequency in population can be helpful for future management; patient who doesn’t has an antigen on his RBCs can produce alloantibody against RBCs, consequently transfusion of present antigen to this patient can cause alloimmunization, it helps to supply antigen negative compatible RBC more easily especially for patient with development multiple antibodies, we also can make a data bank of antigens frequency in our volunteer donors for preparation of reagents panels cells using in antibody detection and identification, useful also in genetic linkage analysis.7,8 Produce alloantibodies against red blood cell antigens can cause immediately or delayed hemolytic transfusion reaction.2 The criteria for selection of donor cells have to focuses on negative of antigens on donor’s cells for the antibodies that are detected in the patient’s serum.9 Only few studies are available, reported antigens frequencies of ABO, Rh and Kell blood groups in India but not reported in Rajasthani patients. Therefore, this study is the first report on the frequency of ABO, Rh and Kell among different patients in Jaipur city.

Most data on previous literatures have determined European, American and some Asian phenotypes of blood group.6 In this study, we have determined frequencies of these phenotypes in Jaipur. Suggestion for future studies, we hope do phenotype frequencies to be determine in all
Phenotype Frequencies of Blood Group Systems (ABO, Rh, Kell) Among Deferent Patients In Jaipur India

parts of India because great country and a vast with several distinct population.

II. MATERIALS AND METHODS:

A total of 103 samples were collected from deferent patients at Nims and SDM Hospital. This study was approved by ethical committee from Nims University, and an informed consent was obtained from the patients after explaining the purpose of the study. One blood sample with volume of 2 ml was collected from each patient for standard tube EDTA. Laboratory investigations were have done to all the studied patients including: ABO, Rh blood grouping and Du testing have done by (BioVue cassettes based on Column Agglutination Technology), finally antigen profile (Rh- Kell Phenotype) by DiaMed ID-Card (C-c-e-K-Ctl, Monoclonal). A cross sectional study was done in the blood bank at SDM Hospital during the period from April 2016 to August 2017.

III. RESULTS:

Out of 103 deferent patients diagnosed in clinics of Nims and SDM Hospital, some patients received at least two unit of red blood cell transfusion. All samples were screened for ABO, Rh blood grouping, Du testing and antigen profile. Total of 103 patients of deferent diseases 54 (52.5%) were males and 49 (47.5%) were females. The age range was from new borne (few days) to 85 years with the mean age of 23 years. Eighty five patients (83%) of the total number of patients (103) were found to have Rh positive (Figure 1). The most common in distribution of ABO, Rh and Kell blood group system was B group flowed by O then A group of these patients (Table 1, Figure 1). Out of 103 patients, the most antigen frequent was antigen (e) which is detected in 90 patients (87.4%) and flowed by D: 82.5%, C: 70.8%, c: 65%, E: 11.6%, while kell antigen was less one, only detected in 3 patients (2.9%) shows on (Table 2).

![Figure 1: Prevalence of Rh antigen in patients](image)

<table>
<thead>
<tr>
<th>Blood group</th>
<th>Total %</th>
<th>Phenotype</th>
<th>Total %</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>24 (23%)</td>
<td>A Positive</td>
<td>18 (17.5%)</td>
<td>A</td>
</tr>
<tr>
<td>B</td>
<td>41 (40%)</td>
<td>B Positive</td>
<td>36 (35%)</td>
<td>B</td>
</tr>
<tr>
<td>AB</td>
<td>11 (10%)</td>
<td>AB Positive</td>
<td>9 (8.7%)</td>
<td>--</td>
</tr>
<tr>
<td>O</td>
<td>27 (26%)</td>
<td>O Positive</td>
<td>21 (20.5%)</td>
<td>O</td>
</tr>
<tr>
<td>Total</td>
<td>103 (100%)</td>
<td>-----------</td>
<td>103 (100%)</td>
<td>--</td>
</tr>
<tr>
<td>Rh</td>
<td>103 (85%)</td>
<td>Positive</td>
<td>85 (83%)</td>
<td>D</td>
</tr>
<tr>
<td>Du test</td>
<td>18</td>
<td>Du+</td>
<td>4 (22%)</td>
<td>--</td>
</tr>
<tr>
<td>Kell</td>
<td>103 (100%)</td>
<td>Positive</td>
<td>3</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td>100</td>
<td>--</td>
</tr>
</tbody>
</table>

![Figure 2: Distribution of ABO, Rh and Kell blood group system](image)

<table>
<thead>
<tr>
<th>Antigen Reference</th>
<th>Present Study (%)</th>
<th>9</th>
<th>8</th>
<th>7</th>
<th>11</th>
<th>11</th>
<th>4</th>
<th>1</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>97.9</td>
<td>95</td>
<td>93</td>
<td>92.7</td>
<td>92.9</td>
<td>95</td>
<td>94.4</td>
<td>93.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>79.9</td>
<td>79.9</td>
<td>79.9</td>
<td>79.9</td>
<td>79.9</td>
<td>79.9</td>
<td>80.0</td>
<td>80.0</td>
<td>80.0</td>
<td></td>
</tr>
<tr>
<td>e</td>
<td>45</td>
<td>52.3</td>
<td>52.0</td>
<td>52.0</td>
<td>52.0</td>
<td>52.0</td>
<td>52.0</td>
<td>52.0</td>
<td>52.0</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>79.4</td>
<td>79.9</td>
<td>79.9</td>
<td>79.9</td>
<td>79.9</td>
<td>79.9</td>
<td>79.9</td>
<td>79.9</td>
<td>79.9</td>
<td></td>
</tr>
<tr>
<td>Kell</td>
<td>1.9</td>
<td>1.9</td>
<td>1.9</td>
<td>1.9</td>
<td>1.9</td>
<td>1.9</td>
<td>1.9</td>
<td>1.9</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>98.0</td>
<td>98.0</td>
<td>98.0</td>
<td>98.0</td>
<td>98.0</td>
<td>98.0</td>
<td>98.0</td>
<td>98.0</td>
<td>98.0</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
IV. DISCUSSIONS

Our study focused on 103 different patients, most patients receive regular blood transfusion. The techniques used were the latest modern sensitive methods (Column Agglutination Technology) to ensure accurate results. Knowledge of various ABO, Rh and Kell blood group antigen and phenotype frequencies in a population is important in future to improve of blood transfusion service. Information in Indian population is limited whereas this is the first study to report the antigens and phenotypes frequencies of various blood group systems in Rajasthani population and first study selected patients, while all previous studies selected donors.

The Rh blood group system is the most polymorphic, and, next to ABO, is the most clinically significant blood group in transfusion medicine.

All of our results have been compared with those from a previous study, and with data from other published articles from India and abroad (Table 2).

In this study, B was the most common blood group followed closely by the O group then A group. This finding is in concordance with other studies published from India [10, (4)]. However, overall worldwide frequency of the B antigen is low, excluding some areas, such as Germany and central Asia and Africa. In studies from Europe, America, and South East Asia, the O antigen has been found to be the most common blood group. While A group the most common in Germany.

The phenotype frequencies in Kell antigen was 2.9% more than in 2% (black) and West Africa 0.8%, but less than from all local & international studies. (4,7,8,9,10)

The worldwide incidence of D antigen is different in different ethnic groups it being 85% in whites and 92% in blacks. (5) In the present study we found D antigen frequency to be 82.5% which is comparable to other studies from India that compatible with studies published from India [10, (4)]. However, overall worldwide frequency of the B antigen is low, excluding some areas, such as Germany and central Asia and Africa. (4) In studies from Europe, America, and South East Asia, the O antigen has been found to be the most common blood group. While A group the most common in Germany. (4)

The phenotype frequencies in Kell antigen was 2.9% more than in 2% (black) and West Africa 0.8%, but less than from all local & international studies. (4,7,8,9,10)

The worldwide incidence of D antigen is different in different ethnic groups it being 85% in whites and 92% in blacks. (5) In the present study we found D antigen frequency to be 82.5% which is comparable to other studies from India that compatible with studies published from India [10, (4)]. However, overall worldwide frequency of the B antigen is low, excluding some areas, such as Germany and central Asia and Africa. (4) In studies from Europe, America, and South East Asia, the O antigen has been found to be the most common blood group. While A group the most common in Germany. (4)

The phenotype frequencies in Kell antigen was 2.9% more than in 2% (black) and West Africa 0.8%, but less than from all local & international studies. (4,7,8,9,10)

The frequency of C antigen in our study was 70.8%, which is low as compared to 78% (in Whites) and higher than 32% (in Blacks) (5) and West Africa 21.9%, but lower than in some studies in India (south Gujarat 91.7% and North India 87%). While C antigen in Cameroon was 95%. (2)

The phenotype frequencies of (c) antigen was found to be positive in 65% of our samples, which is more than in south Gujarat 56.3% and north India 52.8%, but less than 80% (in Whites), 96% (in Blacks) and all international studies. (1,2)

Phenotype (e) antigen was positive in 87.4% in our study, but it is still less than all local and global studies.

Finally, E antigen was present only in 11.6% more than 2% (in Blacks), but lower than all local and global studies.

Our results close to previous study in black group may be Rajasthani population near to black ethnic group. Whatever, our results indicts that Rajasthani population are the best in the world because they don’t have so much from antigens on RBCs, therefore they are good as donor.

V. CONCLUSION

Antigens database on blood donors are little in Raj-India. On the most blood banks the ABO and Rh D antigens are the main examined but other tests are not performed.

Moreover, India is full of its own specificities and multiple ethnic groups that are important to do for better care of patients by improving add tests like phenotyping RBCs of donors at least Rh & Kell, screening and identifying of antibodies in patients.

Our study, first step to make a rare donor antigens database bank and patients’ antibodies also, to provide compatible blood to all multiple blood transfused, alloimmunized patients and enhance safety in transfusion medicine.

REFERENCES

3. P. Rozman et al. “Differentiation of autologous ABO, RHD, RHCE, KEL, JK, and FY blood group genotypes by analysis of peripheral blood samples of patients who have recently received multiple transfusions” Volume 40, August 2000 transfusion.
Adeno-Associated Virus (AAV)-2 Genome in Arthrobacter sp. LS16?


Keywords: AAV; Arthrobacter; Gene annotation

I. INTRODUCTION

In our BLAST analysis against Adeno-associated virus (AAV)-2 genome (GenBank accession no. AF043303.1), we found a 90% homologous sequence of AAV2 in Arthrobacter sp. LS 16 (GenBank accession no. CP012171) published recently [1]. AAV2 is a non-pathogenic single-stranded DNA virus of ~ 4.7 kb in length. The genome of AAV contains a short 145nt of inverted terminal repeats (ITR) flanking the coding region of replication (Rep) and capsid (Cap) genes [2]. The nucleotides in and around the AAV2 integrated sequence of Arthrobacter sp. LS16 was carefully analyzed, annotated and distinguished by their nature and function using standard bioinformatics tools. A region of 4238 bp (3736925-3741162) encoding Rep and Cap genes of AAV2 was found in the Arthrobacter sp. LS16 sequence (Figure 1 and supplementary data file 1). In addition, the upstream region from AAV2 sequence contained partial coding sequence of human alpha subunit of glycoprotein hormone (GLYCA) (154bp, 3736375-3736528), short polyclonal/multiple cloning site (26bp, 3736529-3736554), simian virus 40 polyadenylation signal (242bp, 3736555-3736796), incomplete adenovirus early protein (E1) intron sequence (78bp, 3736847-3736924) and the downstream regions comprised E.coli origin of replication (652bp, 3741177-3741828), neomycin/kanamycin resistance gene (795bp, 3742240-3743034) followed by AAV left ITR (162bp, 3743268-3743429) and cytomegalovirus (CMV) promoter/enhancer (383bp, 3743440-3743822) (Supplementary Table 1). All the foreign components identified (7461bp, 3736375-37363835) in Arthrobacter genome indicates the presence of AAV2 genome along with portions of a shuttle vector plasmid. The two Pac1 restriction sites (3741176, 3743040) commonly used in shuttle vectors for linearization after recombination in competent E.coli cells were preserved exactly at their respective positions [3]. In addition, a non-plasmid GLYCA gene sequence (GenBank accession no. J00152.1) was also identified between a multiple cloning site. It must be noted that GLYCA is used as a quantitative serum expression marker for in vivo studies [4]. To further understand if the presence of foreign DNA sequence in Arthrobacter is mediated by integration elements we screened for the presence of repeat elements. Our analysis revealed two non-identical repeat regions of 249nt (R1) and 127 nt (R2) on both DNA strands in LS16 (Supplementary Table 2) flanking the AAV2 genome. Such a repeat element was also identified in a closely related Arthrobacter sp. YC-RL1 (GenBank accession no. LCYH0000000.1) demonstrating that this feature is a hotspot for integration in Arthrobacter sp. genome. BLASTX search against non-redundant protein databases also revealed the presence of putative conserved domains of Integration Host Factor (IHF) in LS16 (2857050-2857333).

There are many possibilities for the presence of AAV2 based plasmid sequence in Arthrobacter sp.LS16. This sequence may either have naturally integrated or laboratory-induced, both situations that require further detailed analysis of the source samples used for characterization of Arthrobacter sp.LS16. This is important considering that vertical transmission of AAV/antibiotic resistance gene in Arthrobacter has not been reported earlier. More importantly, if the natural integration of AAV genome in Arthrobacter sp.LS16 a common soil bacterium is proven, it may potentially explain the high levels of AAV2 specific neutralizing antibodies (~70%) seen in humans [5].

II. ACKNOWLEDGMENTS

GRJ is supported by an endowment from Joy-Gill Chair (IIT-Kanpur) and research grants from Department of Science of Technology, Government of India (Swarnajayanti Fellowship 2011), Department of Biotechnology (DBT), Government of India (Senior Innovative Young Biotechnologist award 2010: BT/03/IYBA/2010;Grant BT/PR5021/MED/30/757/2012;Grant: BT/PR8599/AGR/36/783/2013 and a Initiation grant (2014-256) from IIT-Kanpur
REFERENCES


Folate Metabolism and Genetic Variant in Down Syndrome: A Meta-Analysis

Ambreen Asim, Sarita Agarwal, Sakil Subhash Kulkarni, Inusha Panigrahi

Department of Genetics, SGPGIMS Lucknow-226014, India
Department of Pediatrics, PGIMER, Chandigarh-160012, India

Abstract— Objectives: Studies investigating the association between gene polymorphisms involved in homocysteine/folate metabolism and Down syndrome (DS) have reported contradictory or inconclusive results. A meta-analysis of 25 studies on association between MTHFR and MTRR polymorphism and DS including 1,934/2,081/cases/controls for MTHFR C677T polymorphism, 1,404/1,632/cases/control for MTHFR A1298C polymorphism and 859/1,132/cases/control for MTRR A66G polymorphism was carried out. Study design: Studies were identified by searching the PubMed database for relevant articles published. Case–control studies were chosen, and odds ratio (OR) with confidence interval (CI) were used to assess the strength of association. Results: The overall results suggested that the variant genotypes MTHFR C677T were associated with DS risk (homozygote, TT vs. CC: OR=2.991; 95% CI: 1.321-3.558; P=0.001 and co dominant model, CT vs. CC: OR=1.1616 (1.216-1.845; P=0.0001). The result of the variant genotypes MTHFR A1298C showed its association with the DS risk (homozygote, AA vs. CC: OR=1.428; 95% CI: 1.016-1.849; P=0.0067).In the stratified analysis, results obtained in variant genotype of MTHFR C677T A66G had increased risk of DS in Caucasian subjects in codominant and dominant model while the increased risk was found in dominant models for Brazilian and Asian subjects. Again, for MTHFR A1298C variant, increased risk was found in Caucasian subject in codominant, dominant and recessive models and in co-dominant model for Brazilian population. The results also show that in A66G variant of MTRR had increased risk of DS in both Caucasian and Brazilian subjects in dominant model. Conclusion: This meta-analysis supports the idea that MTHFR C677T and MTHFR A1298C genotype is associated with increased risk for DS.

Keywords: Down syndrome; Folate metabolism; Gene polymorphisms; Genotype

I. INTRODUCTION

Down syndrome (DS) is one of the commonest disorders with huge medical and social cost. The major cause of DS is the presence of three copies of genes located on chromosome 21. This change is attributed due to abnormal segregation of during meiosis with maternal non disjunction in around 90% of the cases primarily during meiosis I in the maturing oocyte, before conception [1].

The relationships between non disjunction and abnormal folate metabolism have gained interest recent years. The abnormal folate and methyl metabolism lead to DNA hypomethylation and abnormal chromosomal segregation leading to DNA breaks and aberrant chromosome segregation [2,3]. Studies have shown that MTHFR (methylenetetrahydrofolate reductase) and MTRR (methionine synthase reductase) are the two gene identified as one of the risk factors of DS which are involved in folate metabolism [4-10]. Case–control studies that have investigated the association between DS and MTHFR C677T and MTRR A66G polymorphisms have provided inconclusive results. This may be because each study involved small numbers of

II. MATERIALS AND METHODS

Selection criteria
Studies were selected from the PubMed published before 2014 using the suitable keywords. Case reports, editorials and review articles were excluded from this study. Case–control studies that determined the distributions of the MTHFR C677T, MTHFR A1298C and MTRR A66G gene polymorphisms with DS

Data extraction
The following information was extracted from each study: first author, journal, year of publication, “race” of study population, matching, genotyping method, and the number of cases and controls for the MTHFR C677T, MTHFR A1298C and MTRR A66G genotypes. In our
Folate Metabolism and Genetic Variant in Down Syndrome: A Meta-Analysis

IARF International Conference Dubai 12 ISBN: 9788192958093

studies, ethnicities were classified as Caucasians, Asians and Brazilians.

Statistical analysis

The association between MTHFR C677T, A1298C and MTRR A66G gene polymorphisms and DS risk was assessed by using the co-dominant (677CT vs. CC, 677TT vs. CC; 1298AC vs. AA; AG vs. AA, GG vs. AA), the dominant (677CT+TT vs. CC; 1298AC+CC vs. AA; AG+GG vs. AA) and the recessive (677TT vs. CC+CT; 1298CC vs. AA+AC; GG vs. AA+AG) models. The strength of association of the MTHFR and MTRR gene polymorphisms with DS was measured by the ORs together with the 95% CIs. The Chi-square test was first used to assess whether the distribution of genotypes among controls conformed to the Hardy-Weinberg equilibrium (HWE), with P<0.05 considered a departure from HWE.

III. RESULTS

Selection of studies

In this meta-analysis, we have identified 25 studies on association between MTHFR and MTRR polymorphism and DS including 1, 934/2,081/cases/controls for MTHFR C677T polymorphism, 1,404/1,632/cases/control for MTHFR A1298C polymorphism and 859/1,132/cases/control for MTRR A66G polymorphism. For MTHFR C677T polymorphism 18 studies was considered in which 10 studies was from Caucasian population, 2 from Brazil and 4 from Asia. For MTHFR A1298C polymorphism, out of 12 studies, 7 studies were from Caucasian population and 2 and 3 from Brazil and Asia respectively. For MTRR A66G polymorphism, we have included only 7 Inc which 4 from Caucasian population, 2 from Brazil and only 1 from Asia (Table 1 and 2). Meta analysis The meta-analysis of the 18 populations demonstrated that MTHFR C677T was associated with increased DS risk under the homozygote (TT vs. CC: OR=2.991; 95% CI: 1.321-3.558; P=0.001) (Table 3) and co-dominant model (CT vs. CC: OR=1.1616 (1.216-1.845; P=0.0001) (Table 3).

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Country</th>
<th>Author</th>
<th>Year</th>
<th>N</th>
<th>Case/Control for MTHFR C677T</th>
<th>Case/Control for MTHFR A1298C</th>
<th>OR</th>
<th>95% CI</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>USA</td>
<td>Holden et al.</td>
<td>2000</td>
<td>16133</td>
<td>8469</td>
<td>5932</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irish</td>
<td>O’Cofaill et al.</td>
<td>2002</td>
<td>4912</td>
<td>103</td>
<td>2301</td>
<td>4250</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Chang et al.</td>
<td>2005</td>
<td>11120</td>
<td>56</td>
<td>6672</td>
<td>4962</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>Scaloni et al.</td>
<td>2006</td>
<td>9494</td>
<td>359</td>
<td>6091</td>
<td>1957</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brazilian</td>
<td>Brazil</td>
<td>Donadio et al.</td>
<td>2006</td>
<td>19356</td>
<td>3740</td>
<td>1097</td>
<td>2526</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Brazilian</td>
<td>Brazil</td>
<td>Fontenot et al.</td>
<td>2008</td>
<td>23917</td>
<td>4740</td>
<td>13711</td>
<td>6044</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>China</td>
<td>Xie et al.</td>
<td>2014</td>
<td>602</td>
<td>31</td>
<td>485</td>
<td>192</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>China</td>
<td>Zhai et al.</td>
<td>2015</td>
<td>309</td>
<td>485</td>
<td>192</td>
<td>192</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Characteristics of studies included in the meta-analysis for MTHFR gene polymorphisms C677T and A1298C [14-23,26-30].

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Country</th>
<th>Author</th>
<th>Year</th>
<th>N</th>
<th>Case/Control for MTRR A66G</th>
<th>OR</th>
<th>95% CI</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>USA</td>
<td>Holden et al.</td>
<td>2000</td>
<td>16133</td>
<td>8469</td>
<td>5932</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Irish</td>
<td>O’Cofaill et al.</td>
<td>2002</td>
<td>4912</td>
<td>103</td>
<td>2301</td>
<td>4250</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Chang et al.</td>
<td>2005</td>
<td>11120</td>
<td>56</td>
<td>6672</td>
<td>4962</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>Scaloni et al.</td>
<td>2006</td>
<td>9494</td>
<td>359</td>
<td>6091</td>
<td>1957</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Brazilian</td>
<td>Brazil</td>
<td>Donadio et al.</td>
<td>2006</td>
<td>19356</td>
<td>3740</td>
<td>1097</td>
<td>2526</td>
<td>37</td>
</tr>
<tr>
<td>Brazilian</td>
<td>Brazil</td>
<td>Fontenot et al.</td>
<td>2008</td>
<td>23917</td>
<td>4740</td>
<td>13711</td>
<td>6044</td>
<td>31</td>
</tr>
<tr>
<td>Asian</td>
<td>China</td>
<td>Xie et al.</td>
<td>2014</td>
<td>602</td>
<td>31</td>
<td>485</td>
<td>192</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>China</td>
<td>Zhai et al.</td>
<td>2015</td>
<td>309</td>
<td>485</td>
<td>192</td>
<td>192</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Characteristics of studies included in the meta-analysis for MTRR A66G gene polymorphism [14,16,21,24-26,30].

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Country</th>
<th>Author</th>
<th>Year</th>
<th>N</th>
<th>Case/Control for MTHFR C677T</th>
<th>OR</th>
<th>95% CI</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>USA</td>
<td>Holden et al.</td>
<td>2000</td>
<td>16133</td>
<td>8469</td>
<td>5932</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Irish</td>
<td>O’Cofaill et al.</td>
<td>2002</td>
<td>4912</td>
<td>103</td>
<td>2301</td>
<td>4250</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Chang et al.</td>
<td>2005</td>
<td>11120</td>
<td>56</td>
<td>6672</td>
<td>4962</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>Scaloni et al.</td>
<td>2006</td>
<td>9494</td>
<td>359</td>
<td>6091</td>
<td>1957</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Brazilian</td>
<td>Brazil</td>
<td>Donadio et al.</td>
<td>2006</td>
<td>19356</td>
<td>3740</td>
<td>1097</td>
<td>2526</td>
<td>37</td>
</tr>
<tr>
<td>Brazilian</td>
<td>Brazil</td>
<td>Fontenot et al.</td>
<td>2008</td>
<td>23917</td>
<td>4740</td>
<td>13711</td>
<td>6044</td>
<td>31</td>
</tr>
<tr>
<td>Asian</td>
<td>China</td>
<td>Xie et al.</td>
<td>2014</td>
<td>602</td>
<td>31</td>
<td>485</td>
<td>192</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>China</td>
<td>Zhai et al.</td>
<td>2015</td>
<td>309</td>
<td>485</td>
<td>192</td>
<td>192</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: ORs and 95% CI for Down syndrome and the MTHFR C677T polymorphism under different genetic models

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Country</th>
<th>Author</th>
<th>Year</th>
<th>N</th>
<th>Case/Control for MTHFR A1298C</th>
<th>OR</th>
<th>95% CI</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>USA</td>
<td>Holden et al.</td>
<td>2000</td>
<td>16133</td>
<td>8469</td>
<td>5932</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Irish</td>
<td>O’Cofaill et al.</td>
<td>2002</td>
<td>4912</td>
<td>103</td>
<td>2301</td>
<td>4250</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Chang et al.</td>
<td>2005</td>
<td>11120</td>
<td>56</td>
<td>6672</td>
<td>4962</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>Scaloni et al.</td>
<td>2006</td>
<td>9494</td>
<td>359</td>
<td>6091</td>
<td>1957</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Brazilian</td>
<td>Brazil</td>
<td>Donadio et al.</td>
<td>2006</td>
<td>19356</td>
<td>3740</td>
<td>1097</td>
<td>2526</td>
<td>37</td>
</tr>
<tr>
<td>Brazilian</td>
<td>Brazil</td>
<td>Fontenot et al.</td>
<td>2008</td>
<td>23917</td>
<td>4740</td>
<td>13711</td>
<td>6044</td>
<td>31</td>
</tr>
<tr>
<td>Asian</td>
<td>China</td>
<td>Xie et al.</td>
<td>2014</td>
<td>602</td>
<td>31</td>
<td>485</td>
<td>192</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>China</td>
<td>Zhai et al.</td>
<td>2015</td>
<td>309</td>
<td>485</td>
<td>192</td>
<td>192</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: ORs and 95% CI for Down syndrome and the MTRR A66G polymorphism [14,16,21,24-26,30].
The meta-analysis of the 12 populations demonstrated that MTHFR A1298C was associated with increased DS risk under the homozygote (AA vs. CC: OR=1.428; 95% CI: 1.016-1.849; P=0.0067) (Table 4). However, MTRR A66G was not associated with significantly increased risk of DS.

The association of MTHFR (C677T and A1298C polymorphism) and MTRR A66G polymorphism with DS was further stratified by ethnicity. For MTHFR C677T, the positive association was driven by Caucasian codominant model (TT vs. CC: OR=1.709 (1.083, 1.209; P=0.0001) and in Caucasian dominant and recessive model (TT+CT vs. CC: OR=1.167 (1.003-1.354, P=0.0409 and TT vs. CC+CT: OR= 2.084(1.084-2.574;  P=0.0001 respectively). The positive association was also driven by Brazilian dominant model (TT+CT vs. CC: OR=2.925 (1.305-6.031; P=0.0029) and also in Asian dominant model (TT+CT vs. CC: OR=1.563 (1.120-1.970; P=0.001) (Table 3). For MTHFR A1298C polymorphism, the positive association was observed in Caucasian co dominant model (AA vs. CC: 1.583 (0.926-1.560; P=0.3759). Similarly, the positive association was also observed in Caucasian and Brazilian codominant model AC vs. AA (OR=1.280 (1.015-1.591; P=0.0257) and OR= 1.192 (0.942-1.644; P=0.2845). The positive association was also driven by Caucasian population in recessive model (CC vs. AA+AC: OR=1.803 (1.03, 2.509; P=0.0004)) (Table 4). For MTRR A66G polymorphism, the association was observed in both Caucasian and Brazilian population of dominant model (AG+GG vs. AA: (OR=1.533(1.021-2.129; P=0.014**) and (OR= 1.1417(0.999-2.006; P=0.0487) respectively) (Table 5) (Figures 1-3).

Table 4: ORs and 95% CI for Down syndrome and the MTHFR A1298C polymorphism under different genetic models

<table>
<thead>
<tr>
<th>A1298C</th>
<th>AA vs. CC</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>1.428</td>
<td>1.016-1.849</td>
<td>0.0067</td>
</tr>
<tr>
<td>Caucasian</td>
<td>1.167</td>
<td>1.003-1.354</td>
<td>0.0409</td>
</tr>
<tr>
<td>Brazilian</td>
<td>2.925</td>
<td>1.305-6.031</td>
<td>0.0029</td>
</tr>
<tr>
<td>Asian</td>
<td>1.583</td>
<td>0.926-1.560</td>
<td>0.3759</td>
</tr>
</tbody>
</table>

Table 5: ORs and 95% CI for Down syndrome and the MTRR A66G polymorphism under different genetic models

<table>
<thead>
<tr>
<th>A66G</th>
<th>AA vs. AG</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>1.533</td>
<td>1.021-2.129</td>
<td>0.014**</td>
</tr>
<tr>
<td>Caucasian</td>
<td>1.280</td>
<td>1.015-1.591</td>
<td>0.0257</td>
</tr>
<tr>
<td>Brazilian</td>
<td>1.1417</td>
<td>0.999-2.006</td>
<td>0.0487</td>
</tr>
<tr>
<td>Asian</td>
<td>1.1417</td>
<td>0.999-2.006</td>
<td>0.0487</td>
</tr>
</tbody>
</table>

Figure 1: Forest plot of ORs showing TT+CT when compared to the CC genotype. The squares and horizontal lines correspond to the study-specific OR and 95% CI. The area of the squares reflects the study specific weight [14-23,26-30].

Figure 2: Forest plot of ORs showing AA+AC when compared to the CC genotype. The squares and horizontal lines correspond to the study-specific OR and 95% CI. The area of the squares reflects the study specific weight [15-23,28-30].
Folate Metabolism and Genetic Variant in Down Syndrome: A Meta-Analysis

IV. DISCUSSIONS

Folate is an important vitamin that contributes to cell division and growth and is therefore of particular importance during infancy and pregnancy. Folate deficiency during conception and early pregnancy has been associated with slow growth, anemia, weight loss, digestive disorders, and neural tube defects. It has been suggested that certain polymorphism present on folate metabolizing genes can increase a risk of conceive baby with DS. James et al in 1999 have reported a folate metabolizing gene, MTHFR as one of the risk factor for DS babies and have also suggested that a significant increase in plasma homocysteine levels exists in mothers of DS babies [9,11]. MTHFR gene contains 11 exons, located on chromosome 1p36.3 which encodes for an enzyme methylenetetrahydrofolate reductase (MTHFR), a key enzyme in folate and homocysteine metabolism. MTHFR is composed of an N-terminal catalytic and a C-terminal regulatory domain [12]. It catalyzes the biologically irreversible reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which provides the methyl group for the remethylation of homocysteine to methionine [13]. Several single nucleotide polymorphisms are associated with MTHFR including C677T and A1298C which can affect folate and total homocysteine status. The C677T lies at the catalytic domain and the A1298C position is at the regulatory domain. The MTHFR C677T, which involves a cytosine (C) to thymine (T) substitution at position 677 which changes amino acid from alanine to valine in the enzyme. The C677T increases thermolability of MTHFR and causes impaired folate binding and reduced activity of the MTHFR enzyme [14]. The MTHFR A1298C is due to adenine(A) to cytosine (C) transversion at nucleotide 1298, which produces a glutamate to alanine substitution. The A1298C polymorphism in the MTHFR gene has also been associated with decreased enzyme activity [4,5]. MTRR gene is located at 5p15.31 which maintains the methionine synthase enzyme at an active stage for the remethylation of homocysteine to methionine [6]. Methionine synthase reductase regenerates a functional methionine synthase via reductive methylation. Single nucleotide polymorphism in MTRR gene like A66G is found to affect the homocysteine levels in humans [7,8]. The A66G polymorphism in the MTRR gene causes the substitution of isoleucin with the methionine at codon 22 [9]. This polymorphism might be a genetic risk factor for DS since the methionine synthase reaction is important in maintaining normal folate metabolism and DNA methylation. The meta-analysis examined the MTHFR and MTRR gene polymorphism and their relationship to risk of DS. The frequency of 677T and 1298C allele was found to be significantly higher among DS children than the other groups indicating that MTHFR C677T and MTHFR A1298C polymorphism in DS children would be expected to play an important role in bringing about DS. Some studies reported significantly increased prevalence of MTHFR C677T polymorphism as important risk factors for DS babies while in contrast some studies had also reported insignificant association [15-18]. For MTHFR A1298C polymorphism, much more contradictory reports have been presented [16,17,19-25]. Differences in ethnicity may be one reason for controversy and high intake of food folate may neutralize the metabolic impact of MTHFR polymorphism [26].

The T and C allele variant of MTHFR C677T and A1298C respectively may increase the thermolability of MTHFR gene thereby leading to production of abrupt homocysteine levels causing hypomethylation. Hypomethylation leads to abnormal chromosomal segregation which in turns leads to the risk of elevated trisomic fetuses. However this mechanism of abrupt homocysteine causing DNA methylation needs further elucidation and other risk factors must be studied properly on larger case–control study which is the main limitations of the study. The pooled results indicate the homozygous and heterozygous variant of MTHFR C677T polymorphism exerted a risk on DS development (OR=2.991, 95% CI: 1.216-1.845, respectively). However, when the analysis was performed by ethnicity, this was not observed in all subgroups except for Caucasian population (OR=1.709; 95% CI: 1.083-1.209). Similarly, when the study was stratified by ethnicity for dominant model, all three sub group showed increased risk of DS but these effect were reduced overall population of DS (OR=0.7528, 95% CI=0.8115-0.8503).

Figure 3: Forest plot of ORs showing AA+AG when compared to the GG genotype. The squares and horizontal lines correspond to the study-specific OR and 95% CI. The area of the squares reflects the study-specific weight [14,16,21,24-26,30].
Again, the pooled results of MTHFR A1298C polymorphism of homozygous variant exerted a risk on DS development (OR=1.428, 95% CI: 1.016-1.849). However, when the analysis was performed by ethnicity, this was not observed in either of the subgroups. In MTRR A66G polymorphism, reduced risk was observed in all variant but when this risk was stratified in dominant variant, the risk was observed in both Caucasian and Brazilian subgroups (AG+GG vs. AA; OR=1.555, 95% CI: 0.999-2.006, respectively) yet this did not produce any overall effect on dominant model. Further well designed large scale case-control studies might be required to validate the association in different population having the risk of DS.

V. ACKNOWLEDGEMENT

The research was financially supported by the Department of Science and Technology, Ministry of Health, Government of India (DST/Inspire fellowship/2012/499). We are highly grateful to Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, Uttar Pradesh for providing the infrastructure for this research. The acknowledgements are highly credited to Dr. Madhu Khuller, Dr. Ram K Marwaha, Dr Reena Das of the Department of Genetics, SGPGIMS, Lucknow, Uttar Pradesh for providing the blood samples of the patients.

REFERENCES


the risk of Down syndrome pregnancies in young Italian women” by Coppede et al. [2006]. American Journal of Medical Genetics Part A 143A: 1015-1017.


Hallermann Streiff Syndrome-The Oral Manifestations in a Child

Sonu Acharya, Mamta Mohanty, Sheetal Acharya
Institute of Dental Sciences, SOA University, Bhubaneswar, Odisha, India
Professor, Department of Pediatrics, IMS and sum hospitals, SOA University, Bhubaneswar, Odisha
PG Student, Department of Periodontics and Oral Implantology IDS, SOA University, Bhubaneswar, Odisha

Abstract— Hallermann-Streiff syndrome (HSS) is a rare genetic disorder that is primarily characterized by distinctive malformations of the skull and facial region, sparse hair, eye abnormalities, dental defects, atrophic skin changes and a proportionate short stature. Here we discuss a case of 9 years-old female child who presented with abnormal facial features, dental problems and associated cardiac problems

Keywords: Hypoplasia; Aplasia; Syndrome; Orodental; Dysplasia

I. INTRODUCTION

Hallermann-Streiff-François (HSF) syndrome is marked by a characteristic facies with hypoplastic mandible and beaked nose, proportionate short stature, hypotrichosis, microphthalmia with congenital cataract, hypodontia, hypotrichosis, skin atrophy of the face and hypoplasia of the clavicles and ribs [1]. About 15% of cases display intellectual deficit [2]. Neonatal teeth may be present. Upper airway obstruction may result from small nares and glossoptosis (tongue falling backwards) secondary to micrognathia, and these may lead to cor pulmonale [3].

Tracheomalacia is a complication that can lead to chronic respiratory insufficiency, resulting in biventricular cardiac failure and early death. It is a rare clinical entity of unknown etiology that affects growth, cranial development, hair growth, and dental development [4]. It is probably due to a developmental disorder in the 5th-6th gestational week that results in an asymmetric second branchial arch defect [5]. Most cases are sporadic but some have mutations in the GJA1 gene (6q21-q23.2) [6]. Both autosomal dominant and autosomal recessive inheritance have been postulated. Reproductive fitness may be low but rare affected individuals have had affected offspring. Males and females are equally affected. The facies are sometimes described as ‘bird-like’ with a beaked nose. HSS is characterized by seven essential signs, as described by Francois: Dyscephaly (scaphocephaly or brachycephaly with frontal bossing) and typical facies (micrognathia, condylar aplasia, and thin pointed nose); dental anomalies; proportionate nanism; hypotrichosis; atrophy of the skin localized to the head and nose; bilateral microphthalmia; and congenital cataracts. It is known that the dental abnormalities are present in 50%- 80% of the cases; these abnormalities include malocclusion; open bite; severe caries; enamel hypoplasia; supernumerary and neonatal teeth; hypodontia; premature eruption of primary teeth; agenesis of permanent teeth; and maxillary hypoplasia, with poor development of the paranasal sinuses [7]. Our main objective here is to report a case of HSS in 9 years old female child with physical and oral features consistent with the syndrome as well as detection of mutism in this child which has not been reported earlier in cases of HSS.

Case Report

Nine years old female child visited the outpatient department of pediatric dentistry with complain of multiple carious teeth which were asymptomatic. The patient was pre term (34 weeks) low birth weight (2.2 kg) first child of non-consanguineous parents. The parent also gave a history of child having frequent cough and cold for which symptomatic treatment was being given. The mother reported of the child having cardiac problem (ventricular septal defect) for which
surgery had been performed. The mother’s obstetrics’ history did not reveal any systemic disease or drug intake during pregnancy. Physical examination revealed a height of 95 cm (<3rd percentile, <5 Std deviation) with thin built (30 kg) (Figure 1). There was also presence of thin, brittle hairs on scalp (Figure 2). The patient was not able to speak (mute) but she could hear properly. There was no major abnormality detected on routine hematological investigations and ultrasonography of abdomen. Examination of the face revealed frontal bossing, small and thin face, beaked nose with atrophy of skin near nose (Figure 3,4). Intra-oral examination showed all deciduous dentition, multiple caries, enamel hypoplasia (Figure 5). The palate was high arched and V shaped (Figure 6). The orthopantomograph revealed multiple carious deciduous teeth, missing permanent teeth, hypoplastic coronoid and condylar processes (Figure 7). The overall findings suggested Hallermann Streiff syndrome and full mouth rehabilitation was planned. Unfortunately the patient had to be admitted to hospital with acute respiratory distress and did not turn up for dental treatment despite multiple reminders.
II. DISCUSSION

The first record of this syndrome is reported by Aubry in 1893 [8]. Hallermann in 1948 and Streiff in 1950 described patients characterized by “bird face”, congenital cataract, mandibular hypoplasia, and dental abnormalities. The new syndrome was later defined as Hallermann-Streiff Syndrome (HSS), underlining the differences with regard to Franceschetti’s mandibulofacial dysostosis [1]. Variability of clinical signs is typical of HSS. Mostly the cases are sporadic in nature and such inheritance pattern is found. The etiology of the syndrome is unknown but it has been stated that this syndrome results from a developmental disturbance affecting the cephalic ventral extremity at the moment when development of facial bones and of lenses is at the highest degree, thus involving both ectoderm and mesoderm. HSS affects both the sexes equally. According to the literature this syndrome is characterized by seven classical signs, of which our patient had most: i.e., dyscephalia and bird-like face; dental abnormalities; hypotrichosis; atrophy of skin, especially on nose but there were no ocular abnormalities, short stature. Patients with HSS are at high risk of recurrent respiratory tract infections; obstructive sleep apnea; cor pulmonale due to anatomical abnormalities of the upper airway; mandibular hypoplasia; and microstomia. In our case also the patient was reported to have recurrent respiratory infections. Other anomalies reported in such patients are skeletal defects, cardiac defects (ventricular septal defect reported in our patient), hematopoietic abnormalities, and pulmonary anomalies [9]. In addition to dental anomalies, TMJ anomalies have been reported in these patients, including aplasia/hypoplasia of the condyles and coronoid processes, anterior displacement of the condyles, and anterior disk displacements [10]. The hypoplasia of condyles and coronoid processes also was found to be present in our case. The case reported here was found to be mute who has not earlier been reported in this syndrome although no correlation to this syndrome could be established. Orodental anomalies reported in the literature (50-85% of the cases) are microstomia, a small and retracted tongue, mandibular hypoplasia, a high arched palate, class II malocclusion, open bite, hypoplasia of deciduous and permanent teeth, absence of teeth, persistence of deciduous teeth, supernumerary teeth, natal teeth, malformed teeth, and severe and premature caries [10,11]. The orodental abnormalities in the present case (confirmed by radiographic examination) were in the form of severely carious hypoplastic teeth, missing permanent teeth, persistent deciduous teeth, bilateral hypoplastic condyles and coronoid processes. The presence of these abnormalities is of great help in differentiating this condition from oculodentoosseus dysplasia. The differential diagnoses to be considered are progeria (Hutchinson-Gilford), mandibulofacial dysostosis and cleidocranial dysostosis, pyknodysostosis, Franceschetti mandibulofacial dysostosis, ectodermal aplasia and dysplasia, and oculoauriculovertebral dysplasia [12]. The differential diagnosis of HSS from progeria and progeroid syndromes, mandibulofacial dysostosis, and pseudoprogeria is as follows. Progeria differs from HSS by having premature atherosclerosis, nail dystrophy, chronic deforming arthritis, acromicria, and normal ocular findings. Mandibulofacial dysostosis usually has ear anomalies and lower eyelid colobomas [13]. Five negative signs were also described by Francois as differential diagnostic criteria for HSS. These include the absence of (I) auricular anomalies, (II) palpebral anomalies, (III) premature arteriosclerosis, arthrosis, deformities of joints, muscular atrophy, (IV) nail and extremity anomalies, and (V) mental retardation [2]. No negative signs were seen in our patient. Medical treatment in these patients is not necessary after patients reach adulthood,
though some ophthalmologic problems may need attention. The dental problems need thorough treatment with an interdisciplinary approach. Early preventive care protocols, detailed oral hygiene instructions, and regular dental visits are essential for patients with this syndrome

III. CONCLUSION

Despite being a rare syndrome, this syndrome has to be considered in differential diagnosis of other syndromes. Interdisciplinary approach has to be undertaken for the benefit of the patient. Our case had the classical signs of HSS as well as the striking orodental features. The uniqueness of our report is the presence of mutism in this syndrome which has not been reported earlier in literature.

REFERENCES

PARP1 Differentially Interacts with Promoter region of DUX4 Gene in FSHD Myoblasts


[1] Department of Molecular Medicine, George Washington University, Washington DC, USA
[2] Research Center for Genetic Medicine, Children’s National Health System, Washington DC, USA
[3] Department of Integrative Systems Biology, George Washington University, Washington DC, USA

Abstract—Objective: The goal of the study is to identify proteins, which interact with the promoter region of double homeobox protein 4 (DUX4) genes known to be causative for the autosomal dominant disorder Facioscapulohumeral Muscular Dystrophy (FSHD). Methods: We performed a DNA pull down assay coupled with mass spectrometry analysis to identify proteins that interact with a DUX4 promoter probe in Rhabdomyosarcoma (RD) cells. We selected the top ranked protein poly (ADP-ribose) polymerase 1 (PARP1) from our mass spectrometry data for further ChIP-qPCR validation using patients’ myoblasts. We then treated FSHD myoblasts with PARP1 inhibitors to investigate the role of PARP1 in the FSHD myoblasts. Results: In our mass spectrometry analysis, PARP1 was found to be the top ranked protein interacting preferentially with the DUX4 promoter probe in RD cells. We further validated this interaction by immunoblotting in RD cells (2-fold enrichment compared to proteins pulled down by a control probe, p<0.05) and ChIP-qPCR in patients’ myoblasts (65-fold enrichment, p<0.01). Interestingly, the interaction was only observed in FSHD myoblasts but not in the control myoblasts. Upon further treatment of FSHD myoblasts with PARP1 inhibitors, we showed that treatment with a PARP1 inhibitor, 3-aminoenbenzamide (0.5 mM), for 24 h had a suppression of DUX4 (2.6 fold, p<0.05) and ZSCAN4, a gene previously shown to be upregulated by DUX4, (1.6 fold, p<0.01) in FSHD myoblasts. Treatment with fisetin (0.5 mM), a polyphenol compound with PARP1 inhibitory property, for 24 h also suppressed the expression of DUX4 (44.8 fold, p<0.01) and ZSCAN4 (2.2 fold, p<0.05) in the FSHD myoblasts. We further showed that DNA methyltransferase 1 (DNMT1), a gene regulated by PARP1 was also enriched at the DUX4 promoter (p<0.01) and ChIP qPCR validation using patients’ myoblasts (42-fold, p<0.01) but not control myoblasts through ChIP qPCR. Conclusion: Our results showed that PARP1 and DNMT1 interacted with DUX4 promoter and may be involved in modulating DUX4 expression in FSHD.

Keywords: Facioscapulohumeral muscular dystrophy; Myoblast; Immunoblotting; DUX4 Gene

I. INTRODUCTION

Abbreviations: FSHD: Facioscapulohumeral Muscular Dystrophy; DUX4: Double Homeobox 4; RD: Rhabdomyosarcoma; ChIP: Chromatin Immunoprecipitation Assay; qPCR: Quantitative Polymerase Chain Reaction; PARP1: Poly (ADP-ribose) Polymerase 1; 3-AB: 3-Aminobenzamide; ZSCAN4: Zinc finger and SCAN domain containing 4; KOSR: Knockout Serum Replacement

Background

Facioscapulohumeral muscular dystrophy (FSHD) is a digenic disorder and the third most common inherited form of muscular dystrophy [1,2]. The disease is characterized by a progressive and selective weakness and atrophy of the facial, scapular, and humeral muscles followed by weakness of muscles of the lower extremities. The weakness of muscles is often asymmetric [3]. There is currently no pharmacological therapy available to treat this disease.

While clinically indistinguishable, FSHD is sub classified into FSHD1 and FSHD2 based on the genetic causes [4-6]. FSHD1 is genetically linked to contractions of the D4Z4 repeat array at chromosome 4q35 and affects approximately 95% of patients. In patients with FSHD1, the D4Z4 array is contracted from 11-150 repeat units to 1-10 repeat units [4,5]. Individuals who do not have telomeric 4q35 region which contain the D4Z4 array do not develop FSHD [7]. Each of the repeat units contains a conserved ORF of the double homeobox 4 (DUX4) gene [8,9]. The FSHD permissive alleles contain a polyadenylation signal in the pLAM region distal to the repeat array, which allows DUX4 transcripts from the last D4Z4 repeat to be polyadenylated and therefore stabilized for protein translation [10,11]. FSHD2 is not linked to contractions of the D4Z4 repeat array. Recent studies have reported that some of the patients with FSHD2 have mutations in the structural maintenance of chromosomes flexible hinge domain containing 1 (SMCHD1) gene on chromosome 18 [12,13]. In addition, SMCHD1 was shown to be a genetic modifier in FSHD1 [13]. SMCHD1 has
been reported to play an important role in regulating DNA methylation by affecting chromatin structure [14-17]. The mutation of SMCHD1 is believed to contribute to DNA hypomethylation of the D4Z4 region, and derepression of the DUX4 gene. The DUX4 protein is a homeodomain transcription factor that has been shown expressed in germ cells and causes upregulation of genes involved in gametogenesis in affected muscles when aberrantly expressed [18]. Previous studies showed that ectopic expression of DUX4 is cytotoxic both in vitro and in vivo [18-24]. Aberrant expression of DUX4 in muscle cells has been reported to affect molecular pathways involved in myogenesis, oxidative stress responses, immune responses and germ line functions [18-20,23,25,26]. However, how the molecular and cellular changes cause the muscle pathologies and disease phenotypes is not clear. Previous studies reported epigenetic changes of the D4Z4 region in FSHD, including loss of H3K9 trimethylation and HP1 gamma/ cohesin binding, suggesting loss of heterochromatin. In addition, DNA hypomethylation of D4Z4 region in both FSHD1 and FSHD2 was reported, suggesting de-repression of gene expression in the region [27-35]. While the epigenetic mechanisms involved in de-repression of the DUX4 have been studied extensively, the gene regulatory proteins that interact with the DUX4 promoter and regulate the DUX4 expression are not clear.

In this study, we performed a DNA pull-down assay coupled with mass spectrometry to identify proteins that interacted with a DUX4 promoter probe. We further validated interaction between the top ranked protein and the DUX4 promoter using chromatin immunoprecipitation (ChIP). Last, we performed molecular assays using inhibitors against the protein to determine the functional outcome.

II. EXPERIMENTAL PROCEDURES

Cell culture and nuclear extracts preparation

Cell culture and DNA pull-down assay: Rhabdomyosarcoma (RD) cells (ATCC) were cultured to 70% confluence in Dulbecco’s modified Eagle’s medium (Life Technologies) containing 10% heat inactivated fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin (Life Technologies) at 37°C, 5% CO2. Retinal Pigment Epithelium (ARPE-19) cells (ATCC) were cultured to 70% confluence in Dulbecco’s modified Eagle’s medium and F-12 nutrient mixture supplemented with 1% penicillin streptomycin (Gibco) and 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) at 37°C, 5% CO2. We used data generated from the ARPE-19 cells to further narrow down our final protein list to those more specific to muscle cells. To prepare nuclear extracts, cells were first rinsed twice in 1X PBS (Life Technologies), then scraped with a cell scraper and finally collected in 50 ml conical tubes in PBS. The cell suspension was centrifuged at 1000 rpm for 5 min at 4°C and supernatant was removed. The cell pellet was then washed twice with 30 ml of 1X PBS and centrifuged at 1000 rpm and 4°C for 5 min. The packed cell volume (PCV) of pellet was estimated. Subsequently, 5X PCV volume of buffer A (10 mM HEPES (Acros Organics), 1.5 mM MgCl2 (Ambion), 10 mM KCl (Ambion), 2 mM DTT (Sigma-Aldrich), 200 µM PMSF (Sigma-Aldrich) and 1 tablet of protease inhibitor cocktail (PIC) (Roche Applied Sciences) was added to the pellet, mixed well by pipetting and incubated on ice for 10 min. Following incubation, the pellet was homogenized with a hand-held homogenizer. A small aliquot of cell pellet was then mixed with an equal volume of Trypan Blue to assay the extent of homogenization, which was always determined to be complete. The cell lysates were then centrifuged at 4°C for 10 min at 3000 rpm and the supernatant (cytoplasmic extract) was collected and frozen at -80°C. The pellet was centrifuged again at 14000 rpm for 15 min at 4°C and the remaining supernatant was discarded. The nuclear isolates in the pellets were subsequently extracted by adding two volumes of buffer C (20 Mm HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 2mM DTT, 200 µM PMSF, 5 mM NaF and 1 PIC tablet) followed by homogenization and incubation on ice for 30 min. The lysates were centrifuged for 10 min at 4°C and 14000 rpm. Clear supernatant (nuclear extract) was subsequently collected and aliquoted for use in the DNA pull-down assays. The DNA pull-down assay was performed based on a previously described procedure [36]. Briefly, the DUX4 and HTRA1 probes were amplified from human genomic DNA by the Polymerase Chain Reaction (PCR) in the 2720 Thermal Cycler (Applied Biosystems) with GoTaq Green Master Mix (Promega) at the following conditions: 94°C for 5 min and 40 cycles of 94°C for 45 s, 58°C for 30 s, 72°C for 90 s and 72°C for 10 min. The promoter probe of HTRA1 gene is used as control. The following biotin-labeled primers were used in the PCR reaction: DUX4 (forward): 5'-GGGCTGTCCCAGG-3' and (reverse): 5'-GTTCTCCGTGCGGC-3'; HTRA1 (forward) 5'-GAATACG- GACACGCAT-3' and (reverse): 5'-GCCCTGACGTCC-3'. The PCR products were subsequently purified through the QIAGEN Gel Extraction Kit. In the immunoblotting experiment, a random control probe (5'-AGAGTGTCACACTCCCCCTCTG-3') was used.

Nuclear extracts (0.7 mg) from RD cells were incubated with the DUX4 probes, while nuclear extracts from RPE cells were incubated with HTRA1 probes (1.0 µg) in 1 ml binding buffer for 1 h at room temperature. Following the protein-probe binding, samples were incubated with 100 µl of the beads and mixed on a rotating arm at 4°C overnight. The beads were then centrifuged and washed twice in 500 µl of binding buffer with centrifugation performed between each wash step. The beads were then resuspended in 1x LDS sample buffer
Chromatin was electrophoresed on 4-12% Bis-Tris gel (Life Technologies) for 1 h at 100 V. The gel was stained with Coomassie Blue (Bio-Rad) to visualize proteins bands. Whole lanes were then sliced out in 2 mm pieces and processed for in-gel digestion, as described hereafter. Coomassie Blue and SDS were first removed from the gel pieces through sequential washes with 200 µl of water once, and 200 µl of 50% acetonitrile (Sigma-Aldrich) (15 min each) twice. Following removal of liquids, the gel pieces were shrunk immediately with 100 µl of 100% acetonitrile (Sigma-Aldrich) for 5 min. The gel pieces were then rehydrated with 100 µl of 100 mM NH4HCO3 (Sigma-Aldrich) for 5 min. An equal volume of 100% of acetonitrile (Sigma-Aldrich) was added and the gel pieces were incubated in the solution for another 15 min. All liquids were then removed and replaced with 100 µl of 100% acetonitrile (Sigma-Aldrich) until the gel pieces turned white. In-gel digestion was performed by rehydrating dried gel pieces in 20 µl of digestion buffer, consisting of 12.5 ng/µl of trypsin (Promega) in 50 mM of NH4HCO3 (Sigma-Aldrich) for 45 min in an ice bath. Excess digestion buffer was then removed and replaced with 5 µl of 50 mM NH4HCO3 and incubated overnight at 37ºC. The peptides were extracted from the digested gel pieces by incubating the gel pieces with 25 µl of 25 mM NH4HCO3 for 15 min, with occasional vortexing. Equal volumes of 100% acetonitrile (Sigma-Aldrich) were added to each tube for another 15 min, with occasional vortexing. The supernatant was collected and the gel pieces were subjected to two additional extractions with 30 µl of 5% formic acid and 5% of formic acid-acetonitrile (1:1, v/v) for 10 min. The samples were vortexed twice during the incubations. The supernatants from each gel piece were pooled together and dried completely in a vacuum centrifuge. The dried samples were subsequently subjected to Liquid Chromatography-Tandem Mass Spectrometry analysis as previously described [37]. Proteins pulled down by the DUX4 probes were first compared to proteins pulled down only by agarose beads to filter out non-specific interactions. The proteins preferentially interacting with the DUX4 probe were further filtered for nuclear proteins with DNA binding potential. Uniprot Protein Knowledgebase was used to determine the subcellular localization and functions of proteins. Afterwards, results from an independent study were further filtered to limit the list to changes specific to the RD cells in order to retain muscle specific interactions.

Immunoblotting: The proteins pulled down by DNA pull-down assays were separated by electrophoresis on 4-20% SDS-PAGE gradient gels at 100 V for 1 h. They were then transferred onto nitrocellulose at 100 V for 2 h at 4ºC (Life Technologies). The blots were blocked for 1 h in 5% milk (Bio-Rad) prepared in PBS (Life Technologies), 0.1% Tween (Bio-Rad). They were subsequently incubated with anti-PARP1 antibody (Santa Cruz) or anti-DNMT1 antibody (Abcam) at 4ºC overnight. The blots were then washed three times in 0.1% Tween in PBS (Sigma-Aldrich); 5% milk in PBS, and 0.1% Tween in PBS (Life Technologies) for 15 min each, respectively. The blots were then incubated with anti-rabbit HRP (GE Health Sciences) for 2 h at room temperature and then washed in 0.1% Tween in PBS three times for 15 min each. HRP was detected with ECL (Pierce) and chemiluminescence was detected with ECL films (Amersham Pharmacia Biosciences). Cell culture and chromatin immunoprecipitation assay Immortalized human myoblasts were obtained from the Senator Paul Wellstone Muscular Dystrophy Cooperative Research Center at Boston Biomedical Research Institute [38]. The patient myoblast cell line was derived from the biceps of a 42 year old patient with mild muscle weakness (WS157). The control myoblasts were derived from the patient’s 46 year old sibling without FSHD (WS161) [38]. These cells were cultured as previously described [25,37,39]. Briefly, proliferating myoblasts were cultured in a growth medium described previously in detail at 37ºC, 5% CO2 [25]. The culture dish was coated with 0.1% gelatin (Sigma-Aldrich). The medium was changed to growth medium comprising of 15% Knock-Out Serum Replacement (Life Technologies) (KOSR) instead of fetal bovine serum after 72 h of culturing cells. After another 72 h, the cells were harvested and nuclear extracts were prepared. The KOSR was used because it has been shown to increase DUX4 expression [40]. Chromatin was first crosslinked to proteins in 106 human immortalized cells by adding 1% formaldehyde to the cell culture medium. Nuclear extracts were prepared as described previously and chromatin was sonicated to generate fragment sizes of 200-1000 bp using five pulses of 10 s each (10% duty cycle). Sonication was performed using the Sonifier cell disruptor 350 (Branson Sonic Power, Smith Kline Co.) using 3 mm Branson converter. Afterward, the sonicated nuclear lysates were centrifuged. The supernatants were collected and diluted 10- fold in ChiP dilution buffer (EMD Millipore Chemicals) with protease inhibitor cocktail added to the buffer (Roche Applied Sciences). The diluted nuclear lysate was precleared with 75 µl of Salmon Sperm DNA/Protein A Agarose-50% Slurry (EMD Millipore Chemicals) for 30 min at 4°C with agitation and subsequently pelleted by brief centrifugation. The samples were then incubated with 5 µg of rabbit polyclonal anti-PARP1 antibody (Santa Cruz) or DNMT1 antibody at 4°C on a shaker. Rabbit IgG (Abcam) was used as control (5 µg). Samples were then incubated with Salmon Sperm DNA/Protein A Agarose-50% Slurry for 1 h at 4°C with gentle agitation on a shaker to collect the antibody/protein

ISBN: 9788192958093
complexes, which were subsequently pelleted with brief centrifugation. The pellets were sequentially washed with Low Salt Immune Complex Wash Buffer (EMD Millipore Chemicals), High Salt Immune Complex Wash Buffer (EMD Millipore Chemicals), LiCl Immune Complex Wash Buffer (EMD Millipore Chemicals), and TE Buffer (EMD Millipore Chemicals) for 5 min each at room temperature followed by one wash with TE Buffer at 4°C with agitation, and pelleted by centrifugation each time. The DNA was eluted by resuspending the pellets twice in 250 µl of 1% SDS and 0.1 M NaHCO₃ for 15 min with brief centrifugation at the end of each incubation and combining both eluates. Cross-links were reversed by adding 20 µl of 5 M NaCl (Promega) to the combined eluates (500 µl) and incubating for 4 h at 65°C. Then, 10 µl of 0.5 M EDTA (Ambion), 20 µl 1 M Tris-HCL, pH 6.5 (EMD Millipore Chemicals), and 2 µl of 10 mg/ml Proteinase K (Ambion) were added to samples and incubated for 1 h at 45°C. DNA was extracted from the samples using phenol-chloroform extraction and resuspended in 10 µl of water. DNA was amplified in triplicates in SYBR Green PCR Master Mix (Life Technologies) using 1 µM of forward and reverse primers specific to the promoter fragments of each gene and 1 µl of the DNA template in a total volume of 50 µl. The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of amplification using the condition of 95°C for 15 s then 60°C for 1 min. The primers used to amplify the DUX4 promoter fragments were (forward) 5'-ATTCCATGAAGGGGTGGAGCC-3' and (reverse) 5'-TGCAacctcagCGCCGGAC-3'.

**Cell culture and PARP inhibitor study**

A total of 9000 FSHD myoblasts were seeded and cultured in regular media in 25 cm2 flasks according to the protocol described in the previous section. The media was replaced with KOSR containing media 72 h after seeding the cells. The cells were treated with 0.5 mM of 3-AB and fisetin dissolved in DMSO 48 h after changing media for 24 h and subsequently harvested for RNA isolation. RNA isolation, cDNA synthesis, semi-quantitative RT-PCR for DUX4 and real-time qRT-PCR for ZSCAN4 was performed as previously described [25]. Briefly, total RNA was isolated using miRVana kit (Ambion) according to the manufacturer's protocol. The total RNA thus isolated from (3 µg) from each sample was first subjected to DNase I digestion (1 U) to remove genomic DNA contamination as previously described [25]. The RNA was then purified using the RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer’s protocol. Subsequently, the RNA sample was reverse transcribed to cDNA using Superscript III (Life Technologies) and oligo dT primers. The cDNA thus generated was amplified using GoTaq green master Mix (Promega) using 1 µM of forward and reverse primers specific to each gene and 3 µl of cDNA template in a total volume of 20 µl. The thermal cycling conditions included 95°C for 3 min, followed by 40 cycles of amplification using the condition of 95°C for 10 s then 62°C for 45 s. Then further kept for 72°C for 10 min. Primer sequences used for human DUX4 were (forward) 5'-CCCACTCACCAGGACC-3’ and (reverse) 5’ TTCAGAGATGTAACTCTAAATCCA-3’. PCR products were taken from successive PCR cycles and resolved by electrophoresis in 2.0% agarose gel, stained with Ethidium Bromide (EtBr) and visualized under ultraviolet light and imaged using Ingenius Imaging System (Syngene). The samples which were analyzed and compared to each other (internal control, GAPDH and PARP1 inhibitors treated samples) were loaded on the same gel following same settings of the image analyses. Densitometric analysis of EtBr-stained gel bands was performed using Image J software (NIH). To analyze the human zinc finger and SCAN domain containing 4 (ZSCAN4) we amplified the cDNA in triplicates in SYBR Green PCR Master Mix (Life Technologies) using 1 µM of forward and reverse primers specific to each gene and 1 µl of cDNA template in a total volume of 20 µl. The thermal cycling conditions included 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of amplification using the condition of 95°C for 15 s then 60°C for 1 min. Primer sequences used for human zinc finger and SCAN domain containing 4 (ZSCAN4) were (forward) 5'-TGGAAATCTAAGTGCCAAAAA-3’ and (reverse) 5'-CTGCATGTTGGACGTGAC-3’ [24]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control and the primers used were (forward) 5'-TGTCAAGCTCATTTCTCTGTGA-3’ and (reverse) 5'-GTGAGGCTCTCCTTCTTGTG-3’. Standard Ct method was used to calculate expression values relative to GAPDH among the samples (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042380.pdf).

**III. RESULTS**

PARP1 interacts with the promoter of DUX4 in immortalized FSHD myoblasts. To identify proteins that interact with the DUX4 promoter, we performed a DNA pull-down assay coupled with mass spectrometry in RD cells. A 282 bp double stranded DNA probe encompassing 213 bp of the promoter region and 69 bp of the coding region of the DUX4 gene was used (Figure 1A). The selected region contains several DNA regulatory elements including the CATT box, GC box, TACAA box and E box identified by mass spectrometry. The proteins were pulled down by the DUX4 probe were retaining only nuclear proteins.
A total of 12 proteins (Table 1) were identified with poly (ADP-ribose) polymerase 1 (PARP1) ranked at the top (Table 1). No protein that has been reported to interact with the CATT box, GC box, TACAA box, or the E box was identified.

To validate the interaction between the PARP1 and the DUX4 promoter probe, we repeated the DNA pull-down assays using RD cells, followed by immunoblotting assay. Our results demonstrated a 2-fold (p<0.05) enrichment of the PARP1 protein interacting with the DUX4 promoter probe as compared with proteins pulled-down by a control probe with random sequences (Figure 1B). To examine the interaction between PARP1 and DUX4 promoter in human myoblasts, we performed a ChIP assay targeting the promoter region containing a putative PARP1 binding site located at 112-116 bp upstream of the transcription start site [41] using immortalized human myoblasts from an individual with FSHD and myoblasts from the subject’s unaffected sibling as control [37]. A 123 bp amplicon in the DUX4 promoter encompassing the PARP1 binding sites in the middle of the amplicon was designed for ChIP validation as shown in Figure 1A. In the immortalized FSHD myoblasts, real-time quantitative PCR analysis showed a 65-fold enrichment (p<0.01) of the DUX4 promoter fragment in the DNA pulled down by PARP1 antibody as compared with the DNA pulled down by IgG. Interestingly, no significant enrichment of PARP1 was observed in the control myoblasts (Figure 1C). PARP1 inhibition leads to suppression of DUX4 and ZSCAN4 expression To determine functional significance of the interaction between the PARP1 and the DUX4 promoter, we treated FSHD immortalized myoblasts with a commonly used PARP1 inhibitor, 3-aminobenzamide (3-AB). 3-AB mimics the substrate of PARP1, NAD+ and binds PARP1, thereby preventing PARP1 from utilizing NAD+ to poly(ADP-riboseyl)ate (PARylate) targets, which is necessary for PARP1 activity [42,43]. RT-PCR analysis showed 2.6 fold (p<0.05) suppression of DUX4 expression after the immortalized FSHD myoblasts were treated with 0.5 mM 3-AB for 24 h (Figure 2A). We then examined expression of zinc finger and SCAN domain containing 4 (ZSCAN4), one of the germ line genes reported to be dramatically up-regulated by DUX4 previously [18,25,44]. Our results showed a suppression (1.6 fold, p<0.01) of ZSCAN4 expression in response to 3-AB treatment in the FSHD myoblasts (Figure 2B).

Fisetin is a natural compound that was shown to significantly inhibit PARP1 [45]. We examined whether treatment of fisetin will also suppress DUX4 and ZSCAN4 expression in the FSHD myoblasts. RT-PCR analysis showed 44.8 fold (p<0.01) suppression of DUX4 expression after the immortalized FSHD myoblasts were treated with 0.5 mM fisetin for 24 h (Figure 2C). Further, real-time quantitative PCR analysis showed suppression (2.2 fold, p<0.05) of ZSCAN4 expression in response to fisetin treatment (Figure 2D). The data showed that the PARP1 inhibitors, 3-AB and

Table 1: Proteins interacting preferentially with the DUX4 promoter probe

<table>
<thead>
<tr>
<th>Protein symbol</th>
<th>Protein names</th>
<th>Peptide Hits</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARP1</td>
<td>Poly (ADP-ribose) polymerase 1</td>
<td>8</td>
</tr>
<tr>
<td>MSH2</td>
<td>DNA mismatch repair protein</td>
<td>4</td>
</tr>
<tr>
<td>MCM2</td>
<td>DNA replication licensing factor</td>
<td>3</td>
</tr>
<tr>
<td>SFRS5</td>
<td>Serine/arginine-rich splicing factor 5</td>
<td>2</td>
</tr>
<tr>
<td>IF4A3</td>
<td>Eukaryotic initiation factor 4A-III</td>
<td>1</td>
</tr>
<tr>
<td>HNRNL</td>
<td>Metabolon nuclear ribonucleoprotein D-like</td>
<td>1</td>
</tr>
<tr>
<td>IF2B1</td>
<td>Insulin-like growth factor 2 mRNA binding protein</td>
<td>1</td>
</tr>
<tr>
<td>PARP2</td>
<td>Polyadenylate-binding protein 2</td>
<td>2</td>
</tr>
<tr>
<td>TADBP</td>
<td>TAR DNA-binding protein</td>
<td>1</td>
</tr>
<tr>
<td>KC1D</td>
<td>Casein kinase 1 isoform delta SV55N-related matrix-associated</td>
<td>1</td>
</tr>
<tr>
<td>SNF5</td>
<td>adenin-dependent regulator of chromatin subfamily 8 member 1</td>
<td>1</td>
</tr>
<tr>
<td>COP1</td>
<td>Copalomer subunit beta</td>
<td>1</td>
</tr>
</tbody>
</table>

*Figure 1: PARP1 interacts with the DUX4 promoter in FSHD myoblasts. (A) Known and putative genomic and regulatory sequences within the DUX4 promoter probe. The regulatory elements are the CATT box, TACAA box, DBE (D4Z4 binding element), putative PARP1 (Poly[ADP-ribose] polymerase 1) binding site, E box, and ATG (start codon). (B) Immunoblotting was performed using proteins pulled down with the DUX4 promoter probe from nuclear extracts of RD cells. Proteins pulled down with a random probe served as the control for the study. The western blot is representative of four experiments and the densitometry figure shows mean ± SE. *p<0.05 (t-test). (C) ChIP for DUX4 promoter was performed on immortalized FSHD myoblasts and control myoblasts of an unaffected sibling using PARP1 and IgG (control) antibodies (n=3). PARP1 enrichment at the DUX4 promoter in the FSHD myoblasts was calculated by normalizing to the IgG samples. There was no enrichment of PARP1 at the DUX4 promoter in the control myoblasts. The bars represent mean of relative fold-change ± SE. **p<0.01 (t-test).
fisetin, suppressed the expression of DUX4 and its downstream target, ZSCAN4.

**DNMT1 interacts with the promoter of DUX4**

PARP1 has been shown to modulate gene expression by inhibiting the catalytic activity of DNMT1, which leads to DNA de-methylation. Considering that this function involves PARP1 forming a complex with DNMT1 at the promoter of a target gene [46,47], we examined whether DNMT1 interacts with the DUX4 promoter in RD cells using DNA pull-down assay coupled with immunoblotting. The result showed that

![Figure 2: DUX4 and ZSCAN4 are downregulated in response to PARP1 inhibitors, 3-AB and fisetin, in immortalized FSHD myoblasts. Real-time quantitative PCR was performed on FSHD myoblasts treated with 0.5 mM 3-aminobenzamide (A and B) or fisetin (C and D) with untreated cells serving as the control (n=4). Relative expression level of DUX4 (A and C) and ZSCAN4 (B and D) were calculated relative to GAPDH. The bars represent mean of relative expression level ± SE. *p<0.05, **p<0.01 (t-test).](image1)

![Figure 3: DNMT1 interacts with the DUX4 promoter in FSHD myoblasts. (A) Immunoblotting was performed using proteins pulled down with the DUX4 promoter probe from nuclear extracts of RD cells. Proteins pulled down with a random probe served as the control for the study. The western blot is representative of four experiments and the densitometry figure shows mean ± SE. *p<0.05 (t-test). (B) ChIP for DUX4 promoter was performed on immortalized FSHD myoblasts and control myoblasts of an unaffected sibling using DNMT1 and IgG (control) antibodies (n=3). DNMT1 enrichment at the DUX4 promoter in the FSHD myoblasts was calculated by normalizing to the IgG samples. There was no enrichment of DNMT1 at the DUX4 promoter in the control myoblasts. The bars represent mean of relative fold-change ± SE. ***p<0.01 (t-test).](image2)

In this study, the DNA pull-down assay in combination with mass spectrometry analysis was implemented to identify novel gene regulators of DUX4 gene. We used highly stringent criteria to narrow down the identified proteins to 12 proteins. The highest ranked PARP1 was further validated by DNA pull-down assay using the same DNA promoter probe, followed by immunoblotting. We further showed that the interaction between PARP1 and the DUX4 promoter was only detected in FSHD myoblasts but not control myoblasts, suggesting that the DUX4 promoter in the control myoblasts may be inaccessible to the PARP1. This may be due to structural differences of chromatin, which has been shown to be altered in FSHD myoblasts [48-50]. Studies have shown that the D4Z4 region in FSHD patients are associated with loss of the heterochromatin marks H3K9 trimethylation as well as disrupted HP1 gamma/cohesin binding [32,33]. The D4Z4 region has also been shown to be hypomethylated in patients [27-29,34,35]. These altered chromatin patterns on the D4Z4 region in patients may allow interaction between PARP1 and DUX4 promoter in patients’ myoblasts. PARP1 is a chromatin-associated protein involved in the post-translational modification of various nuclear proteins. It functions in various cellular processes such as genomic maintenance, chromatin structure, transcription, replication, cell cycle regulation and cell death through several mechanisms including post-

IV. DISCUSSION

...
translational modification of other proteins with poly (ADP-ribose) chains (PARs), direct interactions with other proteins, and DNA binding [48-60]. PARP1 has been shown to loosen chromatin by modifying proteins attached to chromatin through poly-ADP-ribosylation (PARylation), thereby leading to the formation of localized puffs that permit transcription [61]. In addition, previous studies showed that PARP1 interacted with promoters and affected the gene expression by replacing other nuclear proteins that maintain chromatin structures [62,63]. A recent study suggested that the loss of H3K9 trimethylation pattern may displace SMCHD1 from the D4Z4 region and contribute to increased DUX4 expression [33]. Since we only observed the interaction between the PARP1 and the DUX4 promoter in the FSHD cells but not the control cells, we believe that the shortening of the D4Z4 array created a genomic environment that is more accessible to PARP1.

PARP1 has also been shown to regulate methylation patterns by regulating DNMT1 expression. PARP1 can directly activate DNMT1 transcription as well as regulate its enzymatic function via PARylation [64]. PARP1 was reported to interact and co-localize with DNMT1 at the promoter of target genes. ADP-ribose polymers were added to the DNMT1 by PARP1, which suppresses its ability of methylating the target DNA sequence [42,48,65,66]. DNA de-methylation then leads to activation of the target genes. While patients with FSHD1 and FSHD2 have different primary genetic defects, some epigenetic features are shared in both groups of patients, including changes of histone marks indicating a more open chromatin structure and DNA hypomethylation suggesting active transcription activities in the D4Z4 region [27,28,32,34,35,67]. Our results showed that PARP1 and DNMT1 interacted with the DUX4 promoter, suggesting that this interaction may potentially play a significant role in the demethylation and subsequent upregulation of DUX4 observed in patients with FSHD. PARP1 has been shown to facilitate transcription activation of NF B by acting as a co-activator [68]. It has also been shown increased in inflammatory disorders through NF-KB-dependent gene expression [69]. FSHD has been shown to exhibit inflammatory features in the form of upregulation of both adaptive and innate immune response and the FSHD myoblasts have been shown to be more susceptible to oxidative stress [70-72]. Several PARP1 inhibitors have been shown effective in treating inflammatory disorders. Both PARP inhibitor 3-AB and fisetin have been shown to have anti-inflammatory properties [73-75]. In addition, both agents have also been studied for their anti-oxidative property [76,77]. Considering the function of PARP1 inhibitors and the molecular defects of FSHD, agents with PARP1 inhibiting properties can be investigated further for potential therapeutic applications. In this study, we identified that PARP1 and DNMT1 interact with the DUX4 promoter through immunoblotting. The interactions in FSHD myoblasts were further validated by ChIP. We were unable to detect DNMT1 interaction with the DUX4 promoter through mass spectrometry likely because mass spectrometry is sometimes not sensitive enough to detect presence of proteins that are not very abundant. Immunoblotting has a higher sensitivity of detecting proteins than mass spectrometry, which possibly explains why DNMT1 interaction with DUX4 promoter was detected by immunoblotting but not mass spectrometry. The interaction was then further validated by ChIP. The interaction between PARP1 and DNMT1 and the promoter was further demonstrated in FSHD myoblasts but not the control cells through ChIP assay. It should be noted that there is no difference of PARP1 expression between control and FSHD myoblasts based on previously published profiling studies [10,26,78]. In order to increase DUX4 expression in immortalized FSHD myoblasts during ChIP assay, we cultivated these cells in growth medium containing 15% KOSR instead of 15% FBS. We have recently shown that while DUX4 expression was difficult to be detected in proliferating myoblasts cultured with 15% FBS, it was readily detected in myoblasts cultured with 15% KOSR [79]. This culture condition allows us to study factors that are regulating DUX4 expression. Treatments of PARP1 inhibitors 3-AB and fisetin suppressed the expression of DUX4 and its downstream target, ZSCAN4. Based on previous findings and data from this study, we hypothesized that the alteration of the chromatin due to the contraction of D4Z4 array allows PARP1 to interact with the DUX4 promoter. This may either facilitate the transcription of DUX4 via interacting with other transcription factors or contribute to the DNA hypomethylation in the region by inhibiting DNMT1, which has been proposed to lead to the de-repression of the DUX4 gene. In addition, our study identifies two PARP1 inhibitors, namely 3-AB and fisetin, as novel suppressors of DUX4 expression, and plausible candidates for therapeutic development for FSHD.

Funding
This work was supported by FSH society under award number FSHS- 82013-01 and FSHS-82012-05. FSHD Global Research Foundation, the National Institutes of Health/National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIH/NIAMS) under award number 1R01AR052027. Mass Spectrometry work is partially covered by core NIH grants 2R24HD050846 (National Center for Medical Rehabilitation Research), 5P30HD040677 (Intellectual and Developmental Disabilities Research Center) and UL1RR031988 (CTSI-CN).

Author’s Contribution
VS, SNP, HK, KB, YH and YC made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data. VS and YC
were also involved in drafting the manuscript and revising it critically for important intellectual content.

V. ACKNOWLEDGEMENT

We would like to thank the NIH UMMS Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center for FSHD Research for providing us the immortalized myoblasts.

Author's Information

VS is currently a Postdoctoral Fellow at the Walter Reed Army Institute of Research in the Military HIV Research Program. A portion of this manuscript was part of her doctoral dissertation at the George Washington University, wherein she earned a PhD in Molecular Medicine. SNP and HK are research staffs at Research Center for Genetic Medicine, Children’s National Health System (CNHS). KJB and YH are faculties at George Washington University (GWU) and CNHS and are experts in proteomics studies in skeletal muscles. Y-WC is a faculty at GWU and CNHS with expertise in muscular dystrophies.

REFERENCES


Prenatal Assessment of Three Rare Syndromes from Telangana Region by 3D/4D Sonography


[1] Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Hyderabad, Telangana, India
[2] Modern Government Maternity Hospital, Hyderabad, Telangana, India

Abstract— Ultrasound imaging serves as a powerful tool in the diagnosis of fetal anomalies. The three and four dimensional ultrasound scan overcomes some of the key limitations related to two-dimensional imaging. It facilitates detailed evaluation of suspected fetal abnormalities of face, neural tube, heart, skeletal and many subtle birth defects, which is pertinent to the pediatric surgeon for timely intervention. It also determines the age and developmental stage of the fetus, detects location and abnormalities of placenta, spot abnormal bleeding, ectopic pregnancies. The present article describes the three rare syndromes Meckel Gruber Syndrome, Holt Oram Syndrome (HOS) and Emanuel syndrome identified. During an attempt to screen a total of 3000 high risk pregnant women for the presence of congenital anomalies by 3D/4D sonography prenatally. Disruption of genes due to deletions and translocation are also identified which could be the putative candidate genes in the syndrome onset.

Keywords: Ultrasound; Three-dimensional; Four-dimensional; Fetal anomalies


I. INTRODUCTION

A pregnancy is high-risk or complicated when the life or health of the mother or baby may be at risk. The chances of having an abnormal child with anomalies had been reduced to a greater extent by implementing ultrasonogram in the routine clinical practice. Ultrasound has been used as imaging tool in limited medical practice for more than three decades and had proved useful in the diagnosis of fetal anomalies. The recent advent of 3- and 4-dimensional ultrasonography has facilitated detailed evaluation of suspected fetal abnormalities such as facial anomalies, neural tube defects, heart defects and skeletal malformations, which is pertinent to the pediatric surgeon for timely intervention. Four dimensional ultrasound scan creates a live action images of the unborn child and can determine the age of the fetus, the developmental stage of the fetus, detect uterine placental abnormalities and the location of the placenta, spot abnormal bleeding, ectopic pregnancies and many subtle birth defects. Here we present three rare syndromes diagnosed by 3D/4D sonography prenatally while screening a total of 3000 high risk pregnant women for the presence of congenital anomalies.

Case 1: Meckel Gruber Syndrome (Omim Entry #249000) A routine antenatal sonogram was performed on a 28 year old female presented with G3P2L2D0A0 at 7 month amenorrhea, born to normal parents. The ultrasonogram revealed abnormal morphological features such as echogenic kidneys, occipital encephalocele, club foot, polydactyly (hands and foot) and median cleft lip palate suggestive of Meckel Gruber syndrome (MGS) (Figure 1). It’s a rare lethal disorder, that affects all races and ethnic groups with equal incidence in both sexes and is inherited as an autosomal recessive disorder with an incidence is 1 in 13,250 to 1,40,000 live births worldwide [1]. It is often characterized by occipital encephalocele, polydactyly and bilateral dysplastic cystic (enlarged echogenic) kidneys that may result in oligohydramnios or anhydramnios [2]. The locus for MGS is mapped onto chromosome 17q21-q24 and exhibits some degree of locus heterogeneity [3]. Any mutations or variations in the genes located at this locus cause MGS. The list of candidate genes associated with the studied syndromes is presented in Table 1 while the pedigree of the proband with MGS is given in Figure 2. The proband was advised to undergo Fluorescence In Situ Hybridization (FISH) for trisomies (13, 18 and 21) (Figure 3) and was normal. The parents were counseled regarding the possibilities of several neurological abnormalities, their consequences on the outcome of pregnancy and were advised to take a decision regarding termination.
Case 2: Holt-Oram Syndrome (Omim Entry #142900)

A 24 year old woman presented with G2P1L1D0A0 at 28 weeks of gestation was referred to our institute for antenatal sonogram that showed features of skeletal dysplasia, hands and feet with only 4 digits, single umbilical artery, narrow LV outflow tract, echocogenic focus in LV of heart and dilated loop of bowel, the symptoms suggestive of Holt Oram Syndrome (Figures 4 and 5). It's an autosomal dominant disorder characterized by distinctive malformation of bones of the upper limbs and abnormalities of congenital cardiac and upper-limb malformations frequently occurs and are classified as heart-hand syndromes with the prevalence being 1 in 10,000 births. The females are most commonly affected irrespective of race and ethnic backgrounds [4,5]. The HOS locus is mapped onto chromosome 12q24.21 that carries essential gene/s implicated and its products in the formation of tissues and organs during embryonic development. Any mutations at this locus may lead to variable expression of both cardiac and skeletal defects that have been considered as the chromosomal etiology of this disorder. The phenotypic effects of deletions depend mainly on the size and location of the deleted sequences on the genome that in turn can affect gene dosage (haploinsufficiency) and thus the resulting phenotype [6]. The couple was counseled about the condition and they opted for termination of pregnancy.

Case 3: Emanuel Syndrome (Omim Entry #609029)

A 26 year old woman married to her first cousin, developed a bad obstetric history after the birth of her first child. Her second pregnancy was a pre-term (34 weeks) male child, who died after 3 days of birth. The third and fourth pregnancies resulted in abnormality of rectum and imperforate anus in the new born. She was referred to our unit at
6th month amenorrhea for prenatal diagnosis and counseling (Figure 5). Sonogram revealed a single live fetus of 17-18 weeks with intra-uterine growth retardation, dysplastic ears and congenital heart defects with small VSD along with moderate PDA and PDH confirmed by 2D ECHO were suggestive of Emanuel Syndrome; a rare disorder, first described by a cytogeneticist Dr. Emanuel. It has a distinct phenotype characterized by intrauterine growth restriction, facial dysmorphism, microcephaly, congenital cardiac defects and renal anomalies. Other common birth defects are malformations of anus referred as imperforate anus, where the opening to the anus is missing or blocked and intestinal defect called as diaphragmatic hernia where there is a defect in the muscular wall that separates the lungs and heart from the abdomen or a dimple in the skin just above the buttocks (sacral dimple). This chromosome imbalance consists of either a derivative chromosome 22 [der(22)] as a supernumerary chromosome with the following karyotype: 47,XX,+der (22) t (11;22) (q23;q11) in females or 47,XY,+der (22) t (11;22) (q23;q11) in males rarely [7]. Few cases may even show trisomy of chromosome 22 being inherited from one of the parents, most often the mother. The prevalence of this syndrome is not known, however, literature reports only about 100 cases [8,9]. FISH was performed and showed normal signals for Trisomy 13, 18 and 21 chromosomes (Figure 3) and was normal. The couple was counseled regarding the consequences on the outcome of pregnancy and was advised to take a decision regarding termination. However she continued her pregnancy and delivered a female child with a birth weight of 2.4 Kg associated with the mentioned deformities along with imperforate anus, moderate to large PDA with small mid muscular VSD and ASD. A small dimple was obvious at the lumbo sacral region of spine but meninges were not seen (Figure 4). The child developed acute bilirubin encephalopathy, pneumonia with septic shock and expired at 5 months of age (Table 1).

II. CONCLUSION

The importance of 3D/4D ultra which helps in the diagnosis of the rare disorder is indicated priority towards risk for high morbidity and mortality. Routine ultrasound scan done during pregnancy may

Figure 4: Ultrasonogram of fetus showing symptoms of Holt Oram syndrome.

Figure 5: FISH image along with pre-natal and post-natal images of child with Emanuel syndrome

pick up heart defect or any major birth defect if present but the 3D/4D ultrasound often assist in the study of many anatomical regions like face, extremities, genitalia etc. The prenatal diagnosis of the rare cases mentioned above followed by counselling will help the couple to understand the recurrent risk of syndromes in subsequent pregnancies and help to choose appropriate reproductive options.

Conflict of Interest

None.

III. ACKNOWLEDGEMENT

The authors acknowledge Department of Biotechnology, New Delhi, India for financial support in carrying out the study. We thank all the study subjects for their participation.

REFERENCES


10. RPS6KB1: Ribosomal protein S6 kinase beta-1.

11. SCN4A: Sodium channel protein type 4 subunit alpha.

12. CBX1: Chromobox protein homolog 1.

13. COL1A1: Collagen alpha-1(I) chain.

14. GFAP: Glial fibrillary acidic protein.

15. MAPT: Microtubule-associated protein tau.


18. GALK1: Galactokinase.


22. TBX3: T-box transcription factor TBX3.
Science Vision 2020 on Sulfur Metabolism: What is Needed and What can be Achieved?

KV Venkatachalam
College of Medical Sciences, Nova Southeastern University, Ft. Lauderdale, USA

PAPS synthesis
Many intracellular compounds are sulfonated. The universal sulfonate donor is 3'-phosphoadenosine 5'-phosphosulfate (PAPS). In humans PAPS is synthesized from inorganic sulfate by PAPS synthase (PAPSS) in two steps. First inorganic sulfate combines with adenylate moiety of ATP to form 3'-AdenosinePhosphosolate (APS) by ATP sulfurylase activity of the PAPSS. This enzyme domain activity is one of unique ATP α-phosphoanhydride bond splitting enzyme that is much similar to type I tRNA synthetases. Pyrophosphate which is one of the byproduct is cleaved into two inorganic phosphates. APS in the next step is phosphorylated at the 3'-OH by another molecule of ATP by APS kinase domain of the PAPSS. APS kinase is a β-γ phosphoanhydride bond splitter of ATP that possess Walker motif such as GxxGxxK to form the phosphoryl for transfer reaction. Years ago I was the first to molecularly clone, express, dissect the domain activities of human PAPSS from fetal brain. Currently we are interested in the developmental roles of PAPSS during mice brain development and its clinical correlations. Deficiency of PAPSS2 isoform leads to Spondylo Epimetaphyseal Dysplasia (SEMD) in humans. Thus we are interested in the biochemistry, molecular biology and the clinical relevance of the oxidized form of the sulfur, PAPS the sulfuryl donor in human biology and development.

Methionine Metabolism
Methionine is a sulfur amino acid that has (C-S-C) thioether bond. The sulfur in methionine and cysteine (thiol) forms are in the reduced state. The reductive process of the conversion of sulfate into sulfide and its incorporation into cysteine and eventually methionine are absent in mammals. Mammals obtain methionine mainly through diet which then can be used for s-adenosylmethionine (SAM) and de novo cysteine synthesis. SAM is the universal methyl group donor. The role of SAM in histone, DNA and mRNA methylation is extremely crucial in epigenetic regulations of the genome/genetic expressions. Once the methyl group is transferred to a recipient compounds such as nucleic acids, lipids, proteins and sugars the side product s-adenosyl homocysteine is cleaved into homocysteine and adenosine. Free homocysteine can condense with serine to form cystathionine with the elimination of H2O, catalyzed by pyridoxal phosphate [PLP, (coenzyme derivative of vitamin B6)] dependent cystathionine beta-synthase (CBS) (EC 4.2.1.22). Cystathionine, then can be cleaved by a cystathionine gamma-lyase (CGL) (EC. 4.4.1.1), at trans-gamma position to release cysteine and the rest of the moiety is released as alpha-ketobutyrate and ammonia. The free homocysteine is converted into methionine by methionine synthase synthase [5-methyltetrahydropteroyl- L-glutamate: L-homocysteine S-methyltransferase, (E.C. 2.1.1.13)] using homocysteine, methyl group from 5-methyl-tetrahydrofolate and the coenzyme methylcobalamin (vitamin B12). In bacteria methionine pools are channeled into anabolism by simple formylation of methionine to form formylmethionine (fmet). In the catabolic pathway methionine, can undergo degradation to form methylthiol, a-ketobutyrate and ammonia catalyzed by methionine gammalyase-deaminae (mgld). We have molecularly cloned the methionine degrading enzyme mgld and are looking in to the structure/function aspects of the enzyme. We have also transfected the mgld gene into mammalian cancer cells and we are investigating the cytosolic and various cellular effects. Currently we have localized the mgld in the nucleus of various cancer cell types and asking the question what? global methylation effects would be on the chromatin morphology. Investigation of methylation/demethylation pathways in normal brain and cancer cells

Methylation/demethylation pathways are critical in controlling the epigenetic mode of gene suppression/expression. For the methylation cycle MetS, CBS, SAM synthase and methyltransferase must be tightly regulated. For the demethylation cycle the cytosine methyl hydroxylase, oxidase and the decarboxylase must be tightly regulated. The vitamin B6 dependent cytosine 5-decarboxylase is a poorly understood enzyme that needs to
be characterized thoroughly (Venkatachalam unpublished). Cytosine 5-decarboxylase would be controlled developmentally, temporally and chronologically. De/dysregulated activity of cytosine 5-decarboxylase would result in uncontrolled cell division and metabolism. We plan to investigate the expression and the activities of all the relevant enzymes of methylation/demethylation during normal mice development and in cancer cells during cell division in the presence and absence of transfected mgld gene. The study will help understand the epigenetic control mechanisms that play in neurodegenerative and various debilitating diseases that are regulated by methylation/demethylation patterns.
Role of Genetic Testing in Lung Transplantation; Prediction of Inflammation

[1] Mohamed SA Mohamed
[1] University of Cologne, Deutz-Kalker Str, Cologne, Germany

Abstract—While the genetic matching between the donor and the recipient is essential for the success of the transplant procedure, there are other genetic factors that have the potential to significantly influence the clinical outcome. In this paper, the light is shed on this notion from a relatively new point of view.

Keywords: IL6; IL10; Genetic polymorphisms/mutations; Solid organ transplants

I. INTRODUCTION

Due to the increased incidence of end-stage lung diseases that lead to pulmonary failure, lung transplant becomes a frequent life-saving intervention. Unfortunately, there is a high incidence rate of primary graft dysfunction and failure after transplant. Research is progressing strongly in many directions to improve the clinical outcome of lung transplant. Interleukin-6 (IL-6) is a pro-inflammatory cytokine and an anti-inflammatory myokine. In humans, it is encoded by the IL6 gene [1]. IL-6 is produced mainly by the T cells and the macrophages of the lung, bone marrow, spleen, lymph nodes, brain and skin. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) is the main regulator of IL-6 gene expression, which increases in all cases of tissue injury and inflammation [2]. IL-6 is also secreted by the vascular smooth muscles as a pro-inflammatory cytokine, however, IL-6 processes an indirect anti-inflammatory effects through the antagonization of TNF-alpha and IL-1, and the activation of IL-10 [3]. IL-10 is another cytokine, but with anti-inflammatory actions. It down regulates the expression of cytokines in the T helper-1 cells, and the major histocompatibility class II antigens and costimulatory molecules on the surface of macrophages. Moreover, IL-10 antagonizes the activity of NF-xB [4]. Accordingly, the balance between IL-6 and IL-10 can affect the prognosis of any inflammatory condition, including the ischemic reperfusion injury and the graft-host interaction. Hence, the ratio between both cytokines has the potential to predict the prognosis of lung transplant and the incidence of post-transplant graft failure [5]. A high IL-6/IL-10 ratio post-transplant was found to be associated with severe primary graft dysfunction and 20 fold increased relative risk of death [5].

Clinical and experimental implication of the principle

The Toronto team for lung transplant, which is one of the leading teams in this regard, has already developed a chip to assess the expression levels of mRNAs of certain genes in graft biopsy, as markers for the prognosis of lung transplant. This includes the expression levels of IL-6 and IL-10. This technology is reliable and takes between 20-30 min, which can be performed while the graft on ex vivo perfusion [6]. As the graft biopsies deal with the graft after death of the donor (pre-implantation), in order to assess the expression levels of the IL-6 and IL-10, in addition to other biomarkers, this might be affected by several variables, such as the cause of death of the donor and the associated conditions, the degree of the ischemic reperfusion injury, to which the grafted has been exposed, and can be also attenuated through the application of graft conditioning techniques, such as preconditioning. This might not be able to reflect the post-transplant in vivo graft condition regarding the targeted biomarkers. Moreover, there are imprinted genetic factors that could be considered earlier, by the time the donor makes his decision for donation. Although such imprinted genetic factors might also be reflected during the pre-implantation assessment, it would be much better to identify these risk factors earlier, so that, the precious time and money could be saved. Some of those genetics imprints lie in the mutations/polymorphisms that result in over expression of IL-6 and or defective IL-10.

There are 5 splice variants of IL-6, the standard is a 212 amino acids (aa) and the others are 252aa, 136aa, 189aa, 198aa and 122aa. Certain polymorphisms, such as the homozygous IL6-174G allele, are associated with decreased function of IL-6, reflected as an increased susceptibility to sepsis [7]. Accordingly, polymorphisms and or mutations, associated with the increased IL-6 expression, will ultimately result in high IL-6/IL-10 ratio and the subsequent primary graft dysfunction, whereas those...
associated with defective IL-6 expression would have the potential to provide a safer donor graft.

On the other side, loss- or decrease-of-function mutations of the IL-10 gene will lead to similar consequences. IL-10 receptor A polymorphisms, rs2228054 and rs2228055, have been reported to give the upper hand to the development of the inflammatory bowel disease [8]. A similar reaction could be expected in the lung graft. Following these principles, two polymorphisms of INF-γ and IL-6 genes were reported to be associated with faster development of chronic lung allograft dysfunction (CLAD) following transplant [9,10]. Moreover, genetic polymorphisms/mutations of the Toll-Like Receptor family have a reported impact on the development of inflammation and rejection/CLAD after lung transplant [11-13]. Although some studies failed to correlate CLAD with the known genetic polymorphisms of TNF-α, TGF-β1, and IL-10, those studies were concentrating on the genetic polymorphisms of the recipients rather than the donor (graft) [14,15].

II. CONCLUSION

As the source of the graft cytokines is mainly the resident leukocytes, a preliminary assessment for the increased IL-6 and/or the decreased/defective IL-10 mutations/polymorphisms (in addition to any other relevant genetic markers of inflammation) in the leukocytes of a blood sample can provide a valuable information about the suitability of the potential donor (Figure 1).

This notion is applicable not only for lung transplant, but also for other solid organs transplants. Accordingly, clinical studies to assess the genetic profile of the genes of interest prior to donation, can provide the evidence based association with the rate of success of the transplant procedure and the frequencies of the post-transplant complications. This could be of great value, at least to mark the future grafts as “relatively high risk”, which would indicate the application of special immunomodulatory interventions during lung transplant according to the present recommendation and the reported experiences.

Conflicts of Interest

The intellectual properties included in this manuscript belong solely to the author. Reproduction or use of any of them requires the written permission of the author. No funding was provided for the development of this work. The author welcomes funding cooperation for experimental and clinical studies.

REFERENCES


Toll-like receptors and calcium-activated chloride/potassium channels as immunomodulators of allergic airway inflammation and asthma: a public health research experience in State of Nebraska, USA

[1] [2] Saumya Pandey (Ph.D.)
[1] Department of Biomedical Sciences, Creighton University, Omaha, Nebraska, USA (formerly)
[2] Ajanta Hospital and IVF Centre, Lucknow, Uttar Pradesh, India (formerly)
drasaumyapandey6@gmail.com

Abstract— Allergic airway inflammation and asthma have emerged as major public health problem in the Midwest, United States of America. Targeting Toll-like receptor (TLR) signaling and Calcium-activated Chloride (ClCa)/Potassium (KCa) Channels in unraveling the cellular/molecular mechanisms underlying genetic susceptibility to inflammatory diseases in specific human patient population subset(s) is emerging as an attractive immunotherapeutic pharmacological strategy in management/prevention of asthma. Objectives: My pilot study aimed to investigate the role of TLRs and ClCa/KCa ion channels in human bronchial epithelial cells (NHBEC, BEAS-2B from ATCC), and eosinophils-derived from asthma patients of North American ethnicity.

Methods: Whole cell patch clamp electrophysiological recordings were conducted for ClCa and KCa currents in cultured cells (passages P3-P7) grown on cover-slips. RNA and Protein were extracted from NHBECs, BEAS-2B and eosinophils using Trizol and RIPA methods. Pipette and bath solutions for electrophysiology were subjected to stringent pH and osmolality checks prior to these experiments.

Index Terms- Asthma, Biomarkers, Calcium activated Chloride/Potassium Channels, Toll-like receptors

I. INTRODUCTION

Allergic airway inflammation and asthma have emerged as major public health problem in the Midwest, United States of America. Allergic asthma is one of the most common chronic conditions in Western world, including the State of Nebraska; the clinical sequelae in allergic airway inflammation/asthma include goblet cell metaplasia, mucus hypersecretion, bronchial hyper-responsiveness, smooth muscle hypertrophy, reactive oxygen species generation, eosinophil infiltration, airway obstruction, NF-KB activation and production of inflammatory cytokines [1]. Toll-like receptors, a family of evolutionarily conserved pathogen recognition receptors, initiate inflammatory responses to foreign pathogens; thirteen TLRs are known till date (TLR1-13) and their complex signaling mechanisms involve various intermediaries in the signal transduction pathway for an inflammatory/immune response in the target cell [2]. Calcium-activated chloride channels (CaCC) are primarily expressed in excitable/non-excitable cells, and their functional roles are defined by anion selectivity, activated by intracellular calcium and modulated by Calmodulin kinase (CaMK) II and calcineurin; chloride channels are involved in diverse physiological processes viz. cell migration, proliferation and apoptosis and interestingly, the immunobiological significance of CACC in asthma pathophysiology has been demonstrated recently [3]. Ion channel electrophysiology is an intriguing research area with immense immunotherapeutically relevant clinical/public health impact in deciphering the intricacies of aberrant physiological conditions and/or human diseases. Calcium-activated Potassium channels (KCa) are broadly of three classes: the large conductance (BKCa), intermediate conductance (IKCa) and small conductance (SKCa), and KCa inhibitors have been implicated in human diseases, including asthma, neuronal degeneration and cardiovascular diseases [3]. Racial and ethnic disparities in asthma management and health care utilization are emerging as thrust areas of public health policy-related research in contemporary times; cost-effective predictive research models with biomarkers are warranted to decrease the increasing burden of allergic airway inflammation, atopy and asthma amongst disease-susceptible at-risk individuals of ethnically disparate populations. Surveillance of asthma risk and outcomes-based timeline based strategies may be
developed for prevalence and disease outcome assessments involving risk factors, asthma management, asthma control and prevention. Targeting TLR signaling and Calcium-activated Chloride (ClCa)/Potassium (KCa) Channels in unraveling the cellular/molecular mechanisms underlying genetic susceptibility to inflammatory diseases in specific human patient population subset(s) is emerging as an attractive immunotherapeutic pharmacological strategy in management/prevention of asthma. My pilot study aimed to investigate the role of TLRs and ClCa/KCa ion channels in human bronchial epithelial cells (NHBEC, BEAS-2B from ATCC), and eosinophils-derived from asthma patients of North American ethnicity.

II. METHODS

Cell culture: Human bronchial epithelial cells (NHBEC, BEAS-2B from ATCC), and eosinophils-derived from asthma patients of North American ethnicity were subjected to routine cell culture in a humidified 5% CO2 incubator at 37 degree Celsius; basal media supplemented with 10% Fetal bovine serum (FBS) was utilized for growing cells to 70-80% confluence. MTT assay was performed for cell viability; cell passages P3-P7 were used for pilot experiments. Electrophysiology: Whole cell patch clamp electrophysiological recordings were carried out for ClCa and KCa currents in cultured cells of passages P3-P7 grown on cover-slips; cells were checked for confluence, contamination and viability by microscopically observing their membranes, shape and density, prior to ion channel electrophysiology. Pipette and bath solutions with adjusted Calcium ion concentrations for electrophysiology were freshly prepared and stored at 4 degree Celsius after initial rounds of stringent pH and osmolality checks prior to performing current recordings. Borosilicate patch pipettes were fabricated using the sophisticated Sutter instrument; background noise interfering with current recordings was eliminated, cover-slips with confluent cells were reexamined, and micromanipulators were thereafter adjusted for gigaseal recordings for detection of outwardly and inwardly rectifying chloride and potassium currents.

Molecular biology experiments: RNA and Protein were extracted from NHBECs, BEAS-2B and eosinophils using Trizol and RIPA methods. Nuclease-free water was used for RNA and protein isolation from cell lysates; spectrophotometric determinations of relevant concentrations were performed prior to RT-PCR and Agarose gel electrophoresis for RNA, and Western blot for protein, respectively. The study was duly approved by Institutional Review Board.

III. RESULTS AND CONCLUSIONS

The preliminary findings were convincing and significantly appreciable, considering the study timeline of six months. Cell viability assays demonstrated >80% viability of NHBECs, BEAS-2B cells (ATCC), and eosinophils. The cell-specific membrane dynamics and structural integrity were observed prior to electrophysiologic assessments; cell shape, size and membrane structure were stringently observed, and rigorous pH checks were conducted for measuring accurate, error-free reliable current recordings. Patch clamp electrophysiology recordings detected Chloride and Potassium channel current spikes in adherent cultured cells in presence of intracellular Calcium, and DIDS, anionic Chloride channel inhibitor(s). Inwardly and outwardly rectifying current recordings and subsequent data-curves/plots were reviewed for subsequent software-based analysis. Interestingly, the mean age of clinically diagnosed asthma patients of North American ethnicity (N=7; White N=2, African American N=4, Caucasian N=1) was 47.0 years (s.d. ±5.0 years); the core tenets of good practice research and bioethics, including written informed consent, were followed. Receptor/ion channel molecular biology-based RNA/Protein-related data demonstrated the expression of TLR2 and intermediate conductance IKCa3.1 mRNA transcripts; beta-actin was used as internal control/referent. Experiments were performed in triplicates so as to rule out the possibility of ambiguous/erroneous findings.

TLR2 and TLR4 have been implicated in the pathogenesis of asthma and the inflammatory responses underlying asthmatic exacerbations; TLR4 detects Gram-negative bacteria through their lipopolysaccharides (LPS); while TLR2 plays a pivotal role in recognizing Gram-positive bacteria [2]. Further, TLR2 promotes Th2-biased immune responses, which may be correlated to the Th1/Th2 imbalance in asthma. Genetic polymorphisms affect susceptibility, severity, and responsiveness of asthmatic patients to specific allergens; oxidative stress contributes to the sensitization of allergens by generating an enhanced allergic immune response [4], thus exacerbating the development of allergic asthma.

TLR-based immunotherapeutics and Calcium activated Chloride and Potassium channels alongwith TLR agonists and/or antagonists as well as ion channel inhibitors are emerging as burning areas of asthma research in contemporary times; interrelated immune/inflammation-related biochemical signaling cascades may be targeted for eventual design of immunomodulatory and anti-inflammatory drugs. The immunobiological role of TLRs has been well-documented, and polymorphisms of the TLR genes can result in significant alterations in the severity and susceptibility of respiratory inflammatory diseases amongst disease-susceptible populations of varying genetic landscapes. Recent studies strongly implicate the intriguing role of TLRs in allergic airway inflammation and asthma; TLR2 activation by its synthetic ligand Pam3Cys, in contrast to the activation of TLR-9 by immunostimulatory DNA, induces a prominent Th2-biased immune response
and aggravates experimental asthma [2, 5]. The TLR-4 (Asp299Gly) polymorphism is associated with an increased prevalence of asthma in Swedish children [6]. The promising preliminary findings of my public biomedical research study with a public health perspective implicates the clinical impact of TLR-CiCa/KCa mediated immunomodulation in asthma management. Future gene-epidemiology studies with larger sample size are warranted for development of cost-effective predictive biomarkers for asthma susceptible populations of diverse ethnicities. TLR-CiCa-KCa may eventually prove to be a boon in rationalized asthma gene therapy in the American cohort of State of Nebraska, USA.

REFERENCES


Antimicrobial activity of Terminalia bellerica (Gaertn.) Roxb. against Multi Drug Resistant Staphylococcus aureus

[1] Assistant Professor, Guru Nanak Institute of Pharmaceutical Science and Technology
[2] Associate Professor, Guru Nanak Institute of Pharmaceutical Science and Technology
[3] Associate Professor, Guru Nanak Institute of Pharmaceutical Science and Technology
[4] Professor, Guru Nanak Institute of Pharmaceutical Science and Technology

Abstract: - Present investigation was done in two phases namely isolation and identification of Multi Drug Resistant Bacteria and Antimicrobial activity of Terminalia bellerica Roxb. against the isolated bacteria. Staphylococcus aureus which was isolated from Hospital effluent was found to be resistant to five antibiotics with highest resistance against Cefixime MIC90 24+ 0.00 (µg/ml). Terminalia bellerica, Baheda has an established antimicrobial activity against Gram negative and Gram positive bacteria. S aureus which was proved to be resistant to five different antibiotics was tested for antimicrobial activity of ethanolic extract of T.bellerica outer coat, fruit and seed followed by determination of MIC90. Ethanolic extract of T.bellerica outer coat having highest zone of inhibition was further fractionated and four individual fractions F1 (water), F2 (50% ethanol and water), F3 (ethanol) and F4 (acetone) were tested for antimicrobial activity against isolated Multi Drug Resistant (MDR) S aureus. The fraction which gave highest antimicrobial activity was tested for phytochemical constituents and further purification procedures. T bellerica crude extract of outer coat obtained by maceration gave the highest zone of inhibition of 3.4+0.17(cm). Fraction F4 gave highest zone of inhibition 3.2+ 0.17 (cm) with MIC90 12+0.00(µg/ml).The extractive value and yield of fraction F4 is 1500mg and 0.030%. Phytochemical tests of F4 was found to contain tannin and alkaloids.

Keywords: Antimicrobial, Extractive Value, Multi Drug Resistance, Phytochemicals.

I. INTRODUCTION

Antibiotics are boon to the society as these drugs have potentially reduced the incidence of Infectious diseases and death due to it. Antibiotics can be effective when prescribed correctly and used rationally, but unfortunately the present scenario is different [1]. There is emergence of Antibiotic resistant bacteria which is a present threat to the environment and society. Antibiotic resistance stems from the abuse and irrational use of antibiotics, more over antibiotic resistance among bacteria can spread over several Genera making sensitive bacteria resistant [2]. Some bacteria are resistant to single antibiotic whereas others are resistant to multiple different antibiotics and are often referred to as Multi Drug Resistant Bacteria (MDR). There are several groups of bacteria which has been proved to be a potential MDR such as MRSA (Methicillin Resistant Staphylococcus aureus), Escherichia coli, Haemophilus sp and many other β- Lactamase producer. Pseudomonas aeruginosa is known to have multidrug efflux pump which can forcefully expel nonspecific antibiotics out of the cell whereas Escherichia coli has accumulated many antibiotic resistant gene in a cassette located in an R(resistant) plasmid [3]. The present scenario demands a new antimicrobial agent to combat multidrug resistant bacteria. There are many newly synthesized antimicrobial agents of chemical origin, but with a short life expectancy; hence, herbal products from Indian Medicinal Plants can be an alternative remedy for the present problem. A new lead molecule of herbal origin need to be identified which will be a potential antimicrobial agent or a good resistance modifier [4]. Terminalia bellerica (Gaertn). Roxb. Belongs to “Combretaceae” family and is commonly known as belleric Myrobalan is a fast growing deciduous tree with a rounded crown. It can even grow up to 50 meters [5]. The bark is bluish or ash-grey whereas the inner bark is yellow. Fruits are sub-globular to broadly ellipsoid, light yellow and have brown tomentosa. Leaves are long, alternate, oval and are clustered towards the end of the branch. Flowers are white and yellow in with offensive odour. It is also called as Baheda in India and has been long used in Indian Herbal Medicine. It is anti-helminthic, digestive, laxative and astrigent useful in ailments like cough, diarrhoea, dyspepsia, asthma, bronchitis etc [6]. Many phyto-constituents have been isolated from the fruit such as...
 Anthroquinones, Chebulagic acid, Gallic acid etc; hence, this research is an attempt to study the antimicrobial activity of T. bellerica fruit against Multi Drug Resistant Staphylococcus aureus [7].

II. MATERIALS AND METHODS

SOURCE OF THE PATHOGEN
Effluent from a Dental hospital (Guru Nanak Institute of Dental Science and Research, Sodepur) was collected for the isolation of MDR. Hospital effluent was collected because many antibiotics were applied to the patient during surgery and other treatments which were ultimately discharged into the waste water and; hence, probability of getting a drug resistant pathogen became more.

ISOLATION OF THE PATHOGEN
The sample was serially diluted in 0.9% Saline and plated in Nutrient Agar using Pour Plate Technique for isolation of individual colony. The plates inoculated was further incubated at 37°C for 24 hours. The colonies obtained was further screened for antibiotic resistance [2].

SCREENING OF COLONIES FOR ANTIBIOTIC RESISTANCE
Two well distinguished colonies having opaque white and golden yellow colour respectively were selected for further investigation and; hence, pure cultures were prepared in slant as well as broth. Focus were given to Golden yellow colony as Staphylococcus aureus produces Golden yellow colony and, moreover, there is an increase emergence of MRSA (Methicillin Resistant Staphylococcus aureus) [4].

ANTIBIOTICS SUSCEPTIBILITY PROFILING OF THE ISOLATED COLONIES
The above selected colonies were screened for antibiotic resistance against five following antibiotics namely Azithromycin, Clarithromycin, Amoxicillin, Cefixime and Tetracycline. Considering the MIC90 according to Clinical and Laboratory Standards Institute (CLSI) guidelines 2016, following concentrations of 4µg/ml, 8 µg/ml, 12 µg/ml, 16 µg/ml, 24 µg/ml and 30 µg/ml were prepared. All, the tests were performed in Muller Hinton (HI-Media) Agar Plates [3].

BIOCHEMICAL CHARACTERIZATION OF SELECTED COLONY FOLLOWING BERGEY’S MANUAL
The selected colony which was found to be resistant was subjected to morphological identification using Gram's staining method [2] followed by its biochemical characterization through various Biochemical tests such as Catalase, Oxidase, Glucose Fermentation, and Lactose fermentation, Mannitol Fermentation, Methyl red, Voges proskauer and Indole test. S aureus was finally confirmed by growing in Blood agar and determination of nature of its haemolysis. Pathogenicity of S aureus was determined by performing Coagulase Test. All, the tests were performed according to Standard Bergey’s Manual of Bacteriology to characterize the microorganism accurately [8][23].

PREPARATION OF PLANT EXTRACT
Fresh dry fruit of Terminalia bellerica was collected during November 2014 in the areas of North 24 Paraganas. The fruit was authenticated from Botanical Garden Shibpur Kolkata. The fruit was washed 3-4 times and further dried under shade. The fruit was further crushed and separated into Exocarp (Outer Coat), Endocarp (Fruit) and seed. Each part of the fruit is converted to a fine powder and subjected to extraction.

EXTRACTION PROCEDURE
All the parts of Baheda fruit were extracted by Maceration whereas the Outer Coat was extracted both by Maceration and Ultrasonic Assisted Extraction (UAE) [9], [10].

MACERATION
10g of finely grounded powder was taken and dissolved in 100 ml of 95% Ethanol. The samples were subjected to shaking at 120 rpm at 250°C for 7 days until the extraction was complete. The extract was filtered and evaporated in Rotary Vacuum Evaporator at 450 C until dry. 1 g of ethanol extract was mixed in 5 ml of ethanol to give a concentration of 0.2 mg/ml.

ULTRASONIC ASSISTED EXTRACTION
5g of Baheda Outer coat was taken and suspended in 95% Ethanol in the ratio of 1: 20 w/v. The content was mixed thoroughly and the Ultrasonic bath was set at 250°C for 15 min. Ultra sonication was done for 30 minutes. After the completion of extraction, the extract was centrifuge at 3000 rpm for 15 minutes. The supernatant was collected, filtered and evaporated in Rotary Vacuum Evaporator at 450 C until dry. 1 g of ethanol extract was mixed in 5 ml of ethanol to give a concentration of 0.2mg/ml [10].

DETERMINATION OF ANTIMICROBIAL ACTIVITY OF Terminalia bellerica against MDR Staphylococcus aureus.
Crude extract of Outer coat, fruit, and seed extracted by Maceration and Ultrasonic Assisted extraction was charged against isolated Staphylococcus aureus inoculum volume (0.1 ml) containing 108 cells for determination of antimicrobial activity. The test was performed using standard Disc Diffusion method using sterile paper discs (5mm diameter). The Test Disc with crude extract, Ethanol as Control and Cefixime as Standard were placed in a lawn of S. aureus swabbed in Muller Hinton Agar Plates. Bacterial cultures were further incubated at 370°C for 24 hr. Zone of Inhibitions were measured and all the tests were performed in triplicates and the average zone of inhibition was noted.

IARF International Conference Dubai 45 ISBN: 9788192958093
Antimicrobial activity of Terminalia bellerica (Gaertn.) Roxb. against Multi Drug Resistant Staphylococcus aureus

**PHYTOCHEMICAL STUDY of Terminalia bellerica.**
The extract with the highest zone of inhibition was selected and phytochemical constituents were identified by various phytochemical tests. The yield and extractive value was calculated according to Indian Pharmacopoeia 2014. [11], [12].

**FRACTIONATION of Terminalia bellerica OUTER COAT**
5g of Ethanolic extract of Baheda Outer Coat was absorbed with 10g of Silica, the adsorbed material was washed with 50 ml of Acetone. The Acetone soluble portion was collected and the insoluble portion was washed with 50 ml 100% Ethanol. Ethanol soluble portion was collected and insoluble portion was washed with 50% Ethanol/ water followed by 100% demineralized water. The following fractions were dried and the extractive value, yield of the fractions were further determined [13].

**DETERMINATION OF ANTIMICROBIAL ACTIVITY of Terminalia bellerica FRACTIONS AGAINST MDR Staphylococcus aureus.**
The test was performed using standard Disc Diffusion method using sterile paper discs (5mm diameter). Test Discs with crude extract of all the four fractions, Ethanol as Control and Cefixime as Standard were placed in a lawn of S. aureus swabbed in Muller Hinton Agar Plates and the cultures were further incubated at 370C for 24 hr. Zone of Inhibition was measured and all the tests were performed in triplicates and the average zone of inhibition was noted.

**DETERMINATION OF MIC90 (MINIMUM INHIBITORY CONCENTRATION) of Terminalia bellerica FRACTIONS AGAINST MDR Staphylococcus aureus.**
Each fractions were dissolved in 10% DMSO (Dimethyl Sulfoxide) for the determination of Minimum Inhibitory Concentration of the Extract against the isolated MDR. Concentrations of 4 µg/ml, 8 µg/ml, 12 µg/ml, 16 µg/ml, 24 µg/ml, 30 µg/ml was prepared for all the fractions and the minimum inhibitory concentration was determined against the MDR S.aureus [14].

**DETERMINATION OF PHYTOCHEMICALS ASSOCIATED WITH Terminalia bellerica FRACTIONS.**
Phytochemical tests for all the fractions were performed namely Ferric chloride test and Gelatin test for Tannins, Shinoda test, Ferric chloride test and Zn/HCL reduction test for Flavonoids, Mayer’s test and Wagner’s test for alkaloid, Solubility test and filter paper test for Fats, Ninhydrin test and Biuret test for proteins and Salkowski test for steroid. [11], [12].

**III. RESULTS AND DISCUSSION**

**A. CHARACTERIZATION OF ISOLATED MICROORGANISM FROM HOSPITAL EFFLUENT**

**TABLE I: Biochemical tests done according to Standard Bergey’s Manual**

<table>
<thead>
<tr>
<th>SL NO</th>
<th>BIOCHEMICAL TEST</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CATALASE</td>
<td>Positive</td>
</tr>
<tr>
<td>2.</td>
<td>OXIDASE</td>
<td>Positive</td>
</tr>
<tr>
<td>5.</td>
<td>GLUCOSE FERMENTATION</td>
<td>Negative</td>
</tr>
<tr>
<td>4.</td>
<td>LACTOSE FERMENTATION</td>
<td>Positive</td>
</tr>
<tr>
<td>6.</td>
<td>MANNITOL FERMENTATION</td>
<td>Positive</td>
</tr>
<tr>
<td>7.</td>
<td>VOGES PROSKAERU</td>
<td>Positive</td>
</tr>
<tr>
<td>8.</td>
<td>INDOLE</td>
<td>Positive</td>
</tr>
<tr>
<td>9.</td>
<td>COAGULASE</td>
<td>Positive</td>
</tr>
</tbody>
</table>

**B. DETERMINATION OF PHENOTYPIC ANTIBIOTIC RESISTANCE AGAINST Staphylococcus aureus**

**FIGURE 2:** Comparative study of Standard MIC (Minimum Inhibitory Concentration) from Experimental and Developed MIC from Experimental against MDR S.aureus.
**MIC of 5 Antibiotics against Staphylococcus aureus.**

<table>
<thead>
<tr>
<th>ANTIBIOTICS</th>
<th>SENSITIVE (µg/ml)</th>
<th>INTERMEDIATE RESISTANT (µg/ml)</th>
<th>RESISTANT (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azithromycin</td>
<td>≤ 0.15</td>
<td>-</td>
<td>≥ 0.5</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>≤ 2</td>
<td>16</td>
<td>≥ 2</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>≤ 0.5</td>
<td>4</td>
<td>≥ 1</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>≤ 2</td>
<td>4</td>
<td>≥ 1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>≤ 1</td>
<td>8</td>
<td>≥ 6</td>
</tr>
</tbody>
</table>

**TABLE IV: OVERALL RESISTANCE PATTERN OF ISOLATED MDR S.aureus according to CLSI 2016.**

<table>
<thead>
<tr>
<th>ANTIBIOTICS</th>
<th>EXPERIMENTAL MIC (µg/ml)</th>
<th>STANDARD MIC (µg/ml)</th>
<th>RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azithromycin</td>
<td>0.5</td>
<td>1</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0.5</td>
<td>2</td>
<td>Resistant</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>0.5</td>
<td>2</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.5</td>
<td>4</td>
<td>Resistant</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>12</td>
<td>4</td>
<td>Intermediate Resistant</td>
</tr>
</tbody>
</table>

**TABLE II: Reference range of antibiotics susceptibility of S.aureus according to CLSI Guidelines 2016.**

<table>
<thead>
<tr>
<th>CONCENTRATION OF ANTI BIOTICS (µg/ml)</th>
<th>ZONE OF INHIBITION (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Azithromycin</td>
</tr>
<tr>
<td></td>
<td>Clarithromycin</td>
</tr>
<tr>
<td></td>
<td>Amoxicillin</td>
</tr>
<tr>
<td></td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
</tr>
</tbody>
</table>

C. ANTIMICROBIAL ACTIVITY OF Terminalia bellerica Roxb. AGAINST MULTI DRUG RESISTANT Staphylococcus aureus.

**TABLE III: Average Zone of Inhibition (mm) obtained from charging 5 Antibiotics against Staphylococcus aureus.**
Average zone of Inhibition obtained by performing the experiments in Triplicate.

**FIGURE 4: Antimicrobial activity of Terminalia bellerica crude extract against MDR Staphylococcus aureus.**

**FIGURE 5: Antimicrobial activity of 4 different fractions of crude extract of T.bellerica exocarp.**

<table>
<thead>
<tr>
<th>FRACTIONS</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>F 1</td>
<td>Fraction in water</td>
</tr>
<tr>
<td>F 2</td>
<td>Fraction in 50% ethanol + water</td>
</tr>
<tr>
<td>F 3</td>
<td>Fraction in ethanol</td>
</tr>
<tr>
<td>F 4</td>
<td>Fraction in ace tone</td>
</tr>
</tbody>
</table>

**FIGURE 3: Comparative study of Zone of Inhibition in (mm) of 5 Antibiotics against isolated Staphylococcus aureus.**

**FIGURE 5: Antimicrobial activity of 4 different fractions of crude extract of T.bellerica exocarp.**
Antimicrobial activity of Terminalia bellerica (Gaertn.) Roxb. against Multi Drug Resistant Staphylococcus aureus

**TABLE V: Phytochemical constituents of 4 fractions of Terminalia bellerica Exocarp**

<table>
<thead>
<tr>
<th>FRACTIONS</th>
<th>TANNIN</th>
<th>FLAVONOIDS</th>
<th>ALKALOIDS</th>
<th>FAT</th>
<th>PROTEIN</th>
<th>STEROID</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>F2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F4</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**TABLE VI: Extractive value and Yield of Terminalia bellerica fractions**

<table>
<thead>
<tr>
<th>FRACTIONS</th>
<th>EXTRACTIVE VALUE (mg)</th>
<th>YIELD</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>325</td>
<td>0.065%</td>
</tr>
<tr>
<td>F2</td>
<td>300</td>
<td>0.060%</td>
</tr>
<tr>
<td>F3</td>
<td>350</td>
<td>0.070%</td>
</tr>
<tr>
<td>F4</td>
<td>1500</td>
<td>0.030%</td>
</tr>
</tbody>
</table>

**FIGURE 7: Comparison of MIC of T. bellerica fraction (exocarp) with respect to Standard Cefixime.**

Microscopic examination of the isolated bacteria from effluent was found to be Gram positive Coccus, looked like a bunch of grapes under 100x objective, which was characteristics of Staphylococcus sp (Figure 1). The following microorganism was further characterized by the Biochemical tests according to the Bergey’s Manual. Microorganism was found to be positive for the following tests such as Catalase, Oxidase, Lactose Fermentation, Mannitol Fermentation, Methyl Red, Voges Proskauer, and Indole test (Table I). The above results proved that the microorganism was Staphylococcus aureus. It was further confirmed by growing the bacteria in blood agar showing β haemolysis. The bacteria was found to be pathogenic as it was Coagulase positive as well; hence, it was concluded from the above evidences that the isolated bacterium was none other than a pathogenic Staphylococcus aureus. Antibiotics were charged against the isolated Staphylococcus aureus and the bacteria was found to be resistant against 5 different antibiotics such as Azithromycin, Clarithromycin, Amoxicillin, Cefixime and Tetracycline (FIGURE 2, TABLE IV). The Minimum Inhibitory Concentration (MIC) of the antibiotics were compared with the standard MIC according to CLSI Guidelines 2016 (TABLE 2). The bacteria were proved to be Multi Drug Resistant with the highest resistance against Cefixime MIC90 24+0.00 µg/ml. (TABLE III, TABLE IV, FIGURE 3).

Terminalia bellerica fruit was selected for screening of antibacterial activity against Multi Drug Resistant Staphylococcus aureus and was found to have potent antibacterial activity against isolated MDR S. aureus. Fruit of T. bellerica showed good antimicrobial activity against the MDR and the highest activity was shown by the exocarp.

**FIGURE 6: Comparative Zone of Inhibition Study of all 4 fractions of Terminalia bellerica exocarp taking Cefixime as standard.**
extracted by maceration with zone inhibition of 3.4+0.17 cm (FIGURE 4). The Exocarp extract with the highest antibacterial property was further fractionated to separate the phytochemical constituents responsible for the activity. The acetone fraction of the exocarp gave the highest antimicrobial activity against the isolated MDR with a zone of inhibition of 3.2+ 0.17 cm (FIGURE 5). All, the fractions were tested for phytochemical constituent (TABLE V). Phytochemical constituents present in F4 fraction was Tannin Alkaloid and Steroid. Minimum Inhibitory Concentration of all the fractions against MDR S. aureus was determined and compared with standard Cefixime (with the highest MIC). Comparison of MIC of 4 purified fractions of T. bellerica exocarp with respect to Cefixime showed acetone fraction has the highest antimicrobial activity with the lowest MIC-12 +0 µg/ml and Cefixime with MIC 24+0 µg/ml (FIGURE 5, FIGURE 6). Extractive value and Yield of all the fractions were determined (TABLE VI). P value for the MIC of T. bellerica fractions obtained by ONE WAY ANOVA was P<0.0001 which was statistically significant.

IV. CONCLUSION

Staphylococcus aureus can cause several illnesses ranging from minor skin infection, such as boils, cellulitis, folliculitis, carbuncles to life threatening diseases such as Pneumonia, Meningitis, Osteomyelitis, Toxic Shock Syndrome, bacteremia, and Sepsis. Antibiotics are the only choice for treating those diseases. Unfortunately if Multi Drug Resistant pathogenic Staphylococcus aureus thrives in the environment then it can cause severe form of the above diseases with no fruitful treatment and the ultimate consequence will be increased mortality in population; hence, an alternative remedy is required to combat Multi Drug Resistance. The above study revealed a potent antimicrobial activity of acetone fraction of T. bellerica against isolated MDR S. aureus comprising Alkaloids and tannins as primary phytochemicals. This work can be further proceeded by identification and characterization of the active constituents of T. bellerica exocarp, which can be a lead molecule with antibacterial effect or a good resistant modifier for MDR bacteria. Mechanism of resistance of the above bacteria also need be determined for the formulation of potential therapeutic agent against MDR Staphylococcus aureus.

REFERENCES


Antimicrobial activity of Terminalia bellerica (Gaertn.) Roxb. against Multi Drug Resistant Staphylococcus aureus


